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AVALIAÇÃO DO PAPEL DESEMPENHADO POR VESÍCULAS EXTRACELULARES DERIVADAS DE GLIOBLASTOMA NA MODULAÇÃO DO SISTEMA IMUNE E NA PROGRESSÃO TUMORAL

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Simone de Beauvoir

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PARTE I

RESUMO

O glioblastoma (GBM) é o mais maligno e com a menor taxa de sobrevida dos tumores gliais. O tecido tumoral é composto por muitas células neoplásicas em proliferação, fibroblastos e células do sistema imune. A proliferação tumoral depende de uma rede complexa de fatores, como citocinas, adenosina e vesículas extracelulares (VEs). O papel das VEs ainda é um assunto controverso e sua atividade pró-tumoral ou antitumoral não é totalmente compreendida. Neste contexto, o objetivo desse trabalho foi compreender o papel das vesículas extracelulares derivadas de tumores (VETs), derivadas da linhagem celular C6, na modulação do sistema imune e na progressão do GBM. VETs foram isolados por centrifugação diferencial do sobrenadante da linhagem celular C6. O tamanho e a polidispersão foram analisados por equipamentos Nanosight, ZetaSizer e Microscopia Eletrônica de Transmissão. A estabilidade das VETs foi analisada pelo equipamento ZetaSizer nos dias 1, 4 e 18. Estas vesículas foram caracterizadas pela presença de marcador clássico, como CD9, e também pela presença das enzimas CD39 e CD73. Além disso, as células de GBM C6 foram tratadas com diferentes concentrações de VETs durante 96 h (n = 3) e a viabilidade celular foi avaliada por ensaio MTS. Em seguida, as VETs foram incubadas (8 µg) com linfócitos mesentéricos isolados de ratos Wistar adultos. Após 48 h de incubação, a expressão das enzimas CD39 e CD73 nos linfócitos foi avaliada por citometria de fluxo. Os modelos GBM *in vivo* foram realizados com três grupos de ratos Wistar machos adultos: Controle, coinjeção e imunização. No grupo de coinjeção, as VETs foram coinjetadas com células C6 GBM no estriado por cirurgia estereotáxica. No grupo de imunização, os ratos foram tratados com 20 µg de VETs 10 e 5 dias antes da cirurgia. Após 14 dias de crescimento tumoral, os ratos foram decapitados e o cérebro foi removido para quantificação do volume do tumor e análise do microambiente tumoral. As VETs apresentaram tamanho uniforme (175,2 ± 6,14nm) e a estabilidade, a 4 ° C, durante os 18 dias testados. Os inibidores das enzimas CD39 e CD73 reduziram (52,1% e 57,8%, respectivamente) a formação de ADO, enquanto o efeito do bloqueio do receptor ADO não alterou a concentração deste nucleosídeo. A porcentagem de células viáveis foi significativamente reduzida após o tratamento com 16 e 32 µg / mL de VETs (de 120 ± 2,12% para 82,52 ± 5% e 92,1 ± 7,9%, respectivamente). A incubação de linfócitos T com VETs não alterou a expressão da proteína CD39 e CD73 em nenhum dos subtipos testado de linfócitos T. Além disso, a coinjeção de VETs reduziu o tamanho do GBM de 221 ± 65,1 mm³ para 121 ± 40,6 mm³ em comparação ao controle. O grupo imunização apresentou um menor volume de GBM em comparação ao grupo controle (de 173 ± 91,8 mm³ para 69 ± 20,2 mm³). Buscando entender o mecanismo por trás dessa redução, analisamos a presença de células $CD4^{+}FOXP3^{-}$ e $CD4^{+}FOXP3^{+}$ no microambiente tumoral. O grupo coinjeção mostrou uma redução significativa de células $CD4^{+}FOXP3^{-}$ e $CD4^{+}FOXP3^{+}$, no entanto, não observamos o mesmo no grupo de imunização, que não mostrou diferença entre os grupos. Juntos, nossos resultados sugerem que os VETs desempenham um papel antitumoral na progressão do GBM, reduzindo a proliferação tumoral e a presença de linfócitos T regulatórios no microambiente tumoral.

ABSTRACT

Glioblastoma (GBM) is the most malignant with the poorest survival rate of the glial tumors. The tumoral tissue is composed by many proliferating neoplastic cells, fibroblasts and cells of immune system. Tumor proliferation depends on complex network factors, such as cytokines, adenosine and extracellular vesicles (EVs). The role of EVs remains controversial and their pro-tumoral or antitumoral activity is not fully understand. In this context, the aim of this study was understand the role of tumor-derived extracellular vesicles (TEVs) in the immune system modulation and GBM progression. TEVs were isolated by differential centrifugation of C6 cell line supernatant. Size and polydispersity were analyzed by Nanosight, Zetasizer equipments and Transmission Electron Microscopy. TEVs stability was analyzed by Zetasizer equipment on days 1, 4 and 18. These vesicles were characterized by the presence of EVs classical marker, CD9, also CD39 and CD73 enzymes. Further, C6 GBM cells were treated with different concentrations of TEVs during 96 h (n=3) and cell viability was assessed by MTS assay. Then, GEVs were incubated (8 µg) with mesenteric lymphocytes isolated from adult Wistar rats. After 48 h of incubation, the expression of CD39 and CD73 enzymes was evaluated by flow cytometry. The *in vivo* GBM model was performed with three adult male Wistar rats groups: Control, coinjection and immunization. In the coinjection group, TEVs were coinjecting with C6 GBM cells into the striatum by stereotactic surgery of adult Wistar rats. In the immunization group, rats were treated with 20 µg of TEVs 10 and 5 days before surgery. After 14 days of tumor growth, the rats were decapitated and the entire brain was removed for tumor size quantification and tumor microenvironment analysis. TEVs presented uniform size (175,2±6,14nm) and the stability, at 4°C, during the 18 days tested (186,8±6,64nm). Inhibitors of CD39 and CD73 enzymes reduces (52,1% and 57,8%, respectively) formation of ADO while the effect of ADO receptor blockade did not alter the concentration of this nucleoside. The percentage of viable cells was significantly reduced after treatment with 16 and 32 µg/mL of TEVs (from 120±2,12% to 82,52±5% and 92,1±7,9%, respectively). The incubation of T-lymphocytes with TEVs did not alter the expression of CD39 and CD73 protein in any tested subset of T-lymphocytes. Moreover, the co-injection of TEVs reduces the GBM size from 221 ± 65,1 mm³ to 121± 40,6 mm in comparison to GBM group. Immunization group reduces the GBM size from 173 ± 91,8 mm³ to 69 ± 20,2 mm³. Seeking to understand the mechanism behind this reduction, we analyzed the presence of $CD4^{+}FOXP3^{-}$ and $CD4^{+}FOXP3^{+}$ cells in tumor microenvironment. The coinjection group showed a significant reduction of $CD4^{+}FOXP3^{-}$ and $CD4^{+}$ $FOXP3^{+}$ cells, however we didn't observe the same in immunization group, which has shown no difference between groups. Together, our results suggest GEVs plays an anti-tumoral role in GBM progression by reducing tumor proliferation and presence of T regulatory lymphocytes in tumor microenvironment.

LISTA DE ABREVIATURAS

ADO: Adenosina

Breg: Linfócitos B regulatórios

CD: Células Dendríticas

EXOs: Exossomos

GBM: Glioblastoma

HE: Hematoxilina e eosina

IDH: Isocitrato desidrogenase

ISEV: *International Society of Extracelular Vesicles*

MVs: Microvesículas

NK: *Natural Killer*

OMS: Organização Mundial da Saúde

SNC: Sistema Nervoso Central

Teff: Linfócitos T efetores

Treg: Linfócitos T regulatórios

VEs: Vesículas Extracelulares

VETs: Vesículas Extracelulares derivadas de Tumores

1 INTRODUÇÃO

1.1 Tumores do Sistema Nervoso Central

Os tumores do sistema nervoso central (SNC) correspondem a um amplo grupo, altamente heterogêneo, de doenças que apresentam características clínicas, biológicas e histológicas variadas e correspondem a 2% de todos os tumores malignos do mundo (Ferreira e Rocha, 2004). Em 2018, de acordo com o projeto Globocan, os tumores do SNC apresentaram uma incidência, em nível mundial, de 296.851 novos casos e 241.037mortes (Ferlay et al., 2018). A classificação dos tumores do SNC amplamente adotada é organizada pela Organização Mundial da Saúde (OMS). Nas últimas décadas, a classificação se baseou basicamente em conceitos de histogênese, ou seja, de acordo com características celulares microscópicas e seus níveis de diferenciação, auxiliados pelas técnicas de hematoxilina e eosina (HE) na coloração dessas células e imunohistoquímica. Recentemente, tornou-se claro que essa divisão seria muito simplória dada a complexidade histológica e genética dos tumores. Frente a isso, em 2016, a OMS incorporou parâmetros moleculares na classificação dos tumores do SNC. Com essa mudança, busca-se um diagnóstico e prognóstico mais preciso, bem como um melhor direcionamento para o tratamento (Louis et al., 2016).

Previamente, a OMS agrupava os tumores do SNC nas seguintes categorias: tumores do tecido neuroepitelial, tumores dos nervos cranianos e paraespinais, tumores das meninges, linfomas e neoplasias hematopoiéticas, tumores de células germinativas, tumores da região selar e tumores metastáticos (Louis et al., 2007). A categoria dos tumores do tecido neuroepitelial é composta pelos tumores astrocíticos, também conhecidos como gliomas, os quais compreendem uma variedade de neoplasias que diferem pela sua localização no SNC, idade, sexo, extensão, potencial invasivo, características morfológicas e progressão do curso clínico (Kleihues e Cavenee, 1997). Atualmente, a combinação de características moleculares

e histológicas na classificação dos tumores gerou a necessidade de padronizar a nomenclatura utilizada, a qual passou a consistir da classificação histopatológica seguida pela característica genética.

Os gliomas são os tumores do SNC mais comuns em adultos, originam-se de uma célula glial ou células precursoras e representam, pelo menos, 80% dos tumores cerebrais. Apresentam uma incidência de 3 - 5 por 100.000 pessoas por ano, podendo ocorrer em todas as faixas etárias, sendo mais prevalente em adultos maiores de 45 anos de idade. Os gliomas apresentam características morfológicas e de expressão gênica semelhantes às células gliais. Histologicamente, eles são diferenciados como astrocitomas, oligodendrogliomas, ependimomas e oligoastrocitomas (Furnari et al., 2007; Porter et al., 2010; Preusser, Ribaupierre, & Wo, 2011; Schwartzbaum et al., 2006). Além disso, os gliomas podem ser classificados em tumores de grau I, II, III e IV utilizando critérios estabelecidos pela OMS 2007 (Louis et al., 2007). Os tumores de grau IV, também conhecidos como glioblastoma (GBM), apresentam características mais avançadas de malignidade, incluindo proliferação vascular e necrose, muitas vezes refratárias à radioterapia ou quimioterapia (Cheng et al., 2011; Louis et al., 2007). Na atual classificação da OMS, o GBM foi subdividido em 3 subtipos: GBM, Isocitrato desidrogenase (IDH)-wild type (GBM primário); GBM, IDH-mutante (GBM secundário); e GBM não especificado (IDH não avaliado) (Louis et al., 2016).

1.2 Glioblastoma

O GBM é o mais maligno dos tumores gliais, está associado a uma baixa sobrevida e é caracterizado por grande heterogeneidade genética, morfológica e histológica intratumoral, a qual é composta por muitas células neoplásicas em proliferação, células endoteliais, fibroblastos e células do sistema imune, como macrófagos e linfócitos (Balkwill, 2004; Solinas

et al., 2009). Além disso, o GBM é caracterizado por proliferação celular incontrolada, infiltração difusa, tendência à necrose, angiogênese significativa, resistência a apoptose e diversas alterações genômicas (Furnari et al., 2007; Kesari, 2011; Louis et al., 2007). O GBM pode se manifestar em qualquer idade, mas sua maior incidência encontra-se entre 45 a 85 anos de idade (Nakamura et al., 2007). Estudos realizados pelo CBTRUS (*Central Brain Tumor Registry of the United States*), durante o período de 2008 a 2012, concluíram que o GBM é o segundo tumor primário cerebral mais frequente e o tumor cerebral maligno mais frequente nos Estados Unidos da América (EUA), contribuindo para 15,1% de todos os tumores primários do cérebro e 46,1% dos tumores malignos do cérebro.

O GBM é considerado incurável, com uma expectativa de vida média de 15 meses após o diagnóstico, usando o padrão atual de tratamento, composto de cirurgia de ressecção, radioterapia e quimioterapia. Apesar disso, apenas 3 - 5% dos pacientes sobrevivem 3 ou mais anos, o tratamento continua paliativo para a maioria dos pacientes e a cura se mantém incerta. Os fatores relacionados a essa baixa sobrevida são a barreira hematoencefálica, que limita a entrada de fármacos no SNC, restringindo a terapia e favorecendo o desenvolvimento de resistência (Soffietti, Leoncini, & Rudà, 2007), ao microambiente tumoral e seu um sistema imune deficiente que além de não combater o crescimento neoplásico, pode auxiliar no desenvolvimento tumoral, bem como ao fato do GBM ser um tumor heterogêneo e com alta probabilidade de recidiva. A recorrente recidiva está amplamente relacionada a capacidade infiltrativa e proliferativa desse tumor, impedindo a extinção das células tumorais e células tronco tumorais. Por esses motivos, o GBM apresenta, em geral, um curso rápido e agressivo (Stupp et al., 2009). É importante ressaltar que a proliferação tumoral é dependente de uma rede complexa de fatores, entre eles citocinas, quimiocinas e adenosina, as quais culminam em imunossupressão no microambiente tumoral, orquestrada por células regulatórias, as quais

estão diretamente relacionadas com pior prognóstico em pacientes com GBM (Sayour et al., 2015).

1.3 Microambiente tumoral

O microambiente tumoral representa todas as células tumorais e não-tumorais presentes no tumor, incluindo células do sistema imune como microglia, macrófagos, linfócitos, além de astrócitos, células tronco, fibroblastos, células endoteliais, e uma variedade de moléculas produzidas por essas células que influenciam a progressão tumoral (Schiffer et al., 2018). Já é amplamente conhecido que tumores possuem um infiltrado inflamatório. Apesar desse infiltrado de células inflamatórias variar em tamanho e composição dependendo do tumor, sua presença é uma evidência que apesar do desenvolvimento do tumor pelo organismo, esse busca interferir na progressão tumoral, processo conhecido como vigilância imune. Esse infiltrado está diretamente relacionado com o grau de malignidade do tumor e é sugerido que a presença de linfócitos, macrófagos e microglia no microambiente tumoral é componente indispensável nos processos de proliferação, migração, angiogênese e sobrevivência tumoral (Nieto-Sampedro et al., 2011; Sayour et al., 2015; Watters, Schartner, & Badie, 2005). Nesse âmbito, a modulação do microambiente tumoral pelas células neoplásicas leva a uma série de eventos, como imunossupressão e vascularização, que influenciam a progressão tumoral.

As células imunes presentes no microambiente tumoral incluem as mediadoras da imunidade adaptativa, como linfócitos T e B e células dendríticas, e as efetoras da imunidade inata, como macrófagos e células Natural Killer (NK) (Ferrone & Whiteside, 2007). Os linfócitos T podem ser divididos em linfócito T auxiliar ($CD3^+CD4^+$), T-citotóxico ($CD3^+CD8^+$) e T-regulatório ($CD4^+CD25^{high}FOXP3^+$). Os linfócitos T regulatórios (Treg) são fenotipicamente classificados como $CD4^+CD25^{high}CD39^+FOXP3^+$ e controlam a renovação

celular no timo, regulando a expansão linfocítica, inflamação crônica e processos autoimunes. No câncer, em contrapartida, as células Treg contribuem para um ambiente imunossuprimido e favorecem o desenvolvimento neoplásico, inibindo a ativação de linfócitos T efetores (Teff) e células NK tanto por contato dependente quanto pela liberação de IL-10 e TGF-β (Bastid et al., 2013; Borsellino et al., 2016). Recentemente, também tem sido demonstrada a capacidade de linfócitos B em suprimir células imunes, sugerindo então a denominação linfócitos B regulatórios (Breg) (Rosser & Mauri, 2015). Dessa forma, sabe-se que linfócitos B podem tanto suprimir quanto estimular a resposta efetora do sistema imune, gerando assim resposta anti- ou pró-tumoral, dependendo do sinal recebido (Saze et al., 2013).

Assim, as células inflamatórias presentes no microambiente tumoral podem tanto contribuir para a progressão tumoral quanto podem impedir o desenvolvimento neoplásico. O tumor não só possui mecanismos de evasão imune, mas também é capaz de modificar funções do infiltrado infamatório para criar um microambiente que favoreça a progressão tumoral. Isso é possível devido a capacidade dos tumores de subverter ativamente a imunidade antitumoral por uma variedade de mecanismos, tais como o acúmulo de células Treg capazes de suprimir a imunidade antitumoral, ambiente de hipóxia, via NF-kB que promove liberação de citocinas, entre outros (Denko et al., 2003; T. L. Whiteside, 2013). Gliomas malignos promovem uma série de mecanismos imunomodulatórios que podem resultar na supressão do sistema imune. Nesse contexto, por exemplo, a atividade constitutiva de NF-kB em células cancerígenas está relacionada com a expressão de genes de resistência a apoptose (BCL-xL e survivina), além de marcadores de inflamação (IL1-β, IL-6, COX2). A hipóxia é um potente estimulador de angiogênese e induz produção de citocinas (Nogueira et al., 2011). Células supressoras como, por exemplo, linfócitos Treg podem ser tanto recrutados dos tecidos linfoides (Treg naturais) como podem ser diferenciados de linfócitos T virgens (Treg induzidos) no microambiente dos gliomas (Sayour et al., 2015; Whiteside, 2015).

Estudos recentes mostram que Treg e Breg são capazes de produzir AMP e adenosina, pela presença das enzimas NTPDase1/CD39 e ecto-5'-nucleotidase/CD73 presentes na membrana plasmática, as quais foram capazes de inibir a proliferação de linfócitos Teff (Figueiró et al., 2016; Saze et al., 2013). Essas enzimas estão envolvidas na sinalização purinérgica, responsável por hidrolisar o ATP extracelular até adenosina, uma molécula imunossupressora. O sistema purinérgico é composto por nucleotídeos (como ATP) e por nucleosídeos (como adenosina) que, em geral, têm funções opostas em neoplasias sólidas. O ATP, através da ligação aos seus receptores P2, principalmente o P2X7, pode funcionar como molécula quimiotática para células imunes, além de ser potencialmente tóxico para células neoplásicas (Stagg & Smyth, 2010). Por outro lado, adenosina é descrita como molécula imunossupressora, facilitando o escape dos tumores ao sistema imune através da ligação aos receptores P1, principalmente receptor A2A (Antonioli et al., 2013). No microambiente tumoral, linfócitos regulatórios, que superexpressam a enzima NTPDase1/CD39 (Mandapathil et al., 2009) coordenadamente com células de GBM, que superexpressam ecto-5'-nucleotidase/CD73 (Bavaresco et al., 2008), produzem adenosina pela hidrólise sequencial do ATP extracelular, a qual favorece um ambiente imunossuprimido e pró-tumoral (Xu et al., 2013).

1.4 Vesículas Extracelulares

A comunicação entre células cancerosas e não cancerosas desempenha um papel essencial durante o processo de progressão tumoral e modulação do microambiente tumoral. Whiteside et al (2011) descreveu três principais mecanismos utilizados pelos tumores no escape do sistema imune: acúmulo de células Treg expressando ectonucleotidases, expressão de receptores toll-like e presença de vesículas extracelulares derivadas de tumores. As vesículas

extracelulares (VEs) podem ser divididas em três categorias: exossomos (EXOs), microvesículas (MVs) e corpos apoptóticos. Todas VEs apresentam uma bicamada lipídica; contudo, variam em tamanho, conteúdo e atividade biológica, desde comunicação intercelular (EXOs e MVs) até a renovação celular (corpos apoptóticos) (Kalra, Drummen, & Mathivanan, 2016).

EXOs são pequenas partículas (30 - 150 nm) liberadas por uma variedade de células, como tumorais, embrionárias, dendríticas, macrófagos e células B, T e NK (Anand, 2010; Colombo, Raposo & Théry, 2014; Xie et al., 2010; Zumaquero et al., 2010). Eles se originam do compartimento endossomal das células, em uma fusão de corpos multivesiculares com a membrana plasmática (Colombo, Raposo & Théry, 2014; Schorey & Bhatnagar, 2008; Trams et al., 1981). As MVs, por outro lado, são partículas maiores (100 - 1000 nm) que se originam por exocitose por meio da membrana plasmática (Muralidharan-Chari et al., 2009). Por esse motivo, essas vesículas, especialmente os EXOs, são muito semelhantes às células que as originaram em termos de conteúdo de proteína, além de expressarem uma série de antígenos tumorais quando secretados por células cancerígenas (Parolini et al., 2009). Além disso, essas vesículas possuem um importante papel na comunicação intercelular com funções autócrinas e parácrinas, mediando a regulação e ativação da resposta imune (Montecalvo et al., 2019). Por exemplo, VEs originadas de células T ativadas podem mediar a morte celular induzida por ativação, ao passo que VEs derivados de tumores podem imunizar contra o desafio tumoral, por meio da ativação de um sistema imune antitumoral (Bu et al., 2015; Harshyne et al., 2015) ou inibir o sistema imune pela formação de citocinas e adenosina, criando um ambiente pró-tumoral (Clayton et al., 2011; Liu, Wang, & Yuan, 2013).

Devido a essas características, os VEs derivados de tumores são sistemas eficientes para a transferência in vivo de sinais “cross-talk”, apresentando múltiplas moléculas bioativas associadas que sugerem que eles apresentem um papel central na geração e modulação do

microambiente tumoral (Marhaba et al., 2008; Park et al., 2010) e amplamente discutido na revisão presente na Parte II a seguir. No entanto, apesar de estudos pré-clínicos e mesmo clínicos demonstrarem a capacidade de VEs em modular o sistema imune, é desconhecido até o momento se a modulação das células efetoras antitumorais é por uma ação direta ou pela modulação de linfócitos Treg. Além disso, devido ao fato dessas vesículas conterem antígenos tumorais e moléculas coestimulatórias, já foi mostrado que essas vesículas podem apresentar uma atividade anti-tumoral. Portanto, ainda é limitado o conhecimento sobre as consequências da interação entre sistema purinérgico, VEs e linfócitos regulatórios no microambiente tumoral e, consequentemente, na progressão do GBM.

2 OBJETIVOS

2.1 Objetivo Geral

Entender o papel das VEs derivadas de células de GBM na modulação do sistema imune e na progressão tumoral.

2.2 Objetivos Específicos

- Caracterizar as VETs quanto a expressão das proteínas CD9, CD39 e CD73;

- Avaliar a ativação de linfócitos T periféricos de ratos após a incubação com VETs através dos marcadores CD4, CD8, CD39 e CD73;

- Avaliar a influência das VETs na progressão de GBM implantado em ratos;

- Avaliar a influência das VETs na modulação do microambiente tumoral de GBM implantado em ratos.

PARTE II

3 RESULTADOS

Os resultados estão descritos em dois capítulos, os quais correspondem a uma revisão e um artigo original a serem submetidos para publicação (Capítulo I e II).

3.1 CAPÍTULO I

Extracellular vesicles in cancer progression: are they part of the problem or part of the solution?

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Title: Extracellular vesicles in cancer progression: are they part of the problem or part of the solution?

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Abstract

Extracellular vesicles (EVs) are released by almost all cells. In cancer progression, they modulate tumor microenvironment by interacting with immune cells and carry immunosuppressive and immunostimulatory molecules. This modifying of the tumor environment occurs through different mechanisms and depends on their cargo content, which is broadly defined by their cellular origin, host immune system, cancer type and stage. Hence, there are some conflicting reports regarding the role of EVs in cancer progression. The mainly reports with vesicles bearing immune-activating effects are related to dendritic cells-derived EVs (DEVs), but there are some, further, related with tumor-derived EVs (TEVs). Also, several mechanisms of T cell suppression have been proposed, including enzymatic production of immunosuppressive adenosine by CD39 and CD73 present on EVs.

Keywords: Extracellular vesicles, cancer, purinergic signaling, immune system

1. Introduction

The way by which cells function and communicate is not fully understood. One area of cell function involves the release Extracellular Vesicles (EVs). Regarding this matter, EVs were discovered as signaling particles used by cells to communicate with each other. During cancer progression, this communication occurs with neighboring cancerous and non-cancerous cells, performing an essential role in cancer progression (Andaloussi, Mager, Breakfield, & Wood, 2013). In this context, Whiteside et al. described three mechanisms that tumors can use to escape from the immune system: presence of tumor-derived extracellular vesicles (EVs), accumulation of regulatory T-cells expressing ectonucleotidases and expression of toll-like receptors (TLR) on tumor cells (Whiteside, Mandapathil, Szczepanski, & Szajnik, 2011). Accordingly, an intriguing mechanism of tumor immune escape is through the release of EVs for communication with the neighboring cells. Commonly, EVs have been divided in three categories: exosomes (EXOs), microvesicles (MVs) and apoptotic bodies (ABs). All these subtypes own a lipid bilayer membrane, although varying in size, content and biologic activity, from intercellular communication (EXOs and MVs) to removal of cellular debris (ABs) (Kalra, Drummen, & Mathivanan, 2016). In this review, we will focus in the first two categories (EXOs and MVs).

The first indication suggesting the presence of EVs was made in the 1940s, when a clotting factor was discovered in a platelet-free serum (Chargaff & West, 1946), which was, later,

discovered as lipid-containing particles (Wolf, 1967). Following, in 1970s and 1980s, several studies observed the presence of small vesicles in a variety of body fluids. In the same period, those vesicles were identified in peripheral circulation and malignant effusions in ovarian cancer patients (Pan et al., 1985; Taylor, Homesley, & Doellgast, 1980). Trams et al. (1981), termed a branch of extracellular vesicles as EXOs (Trams et al., 1981)

EXOs are a subpopulation of EVs, secreted by diverse kinds of living cells, ranging in size from 30 to 150 nm; however, without a consensus because some groups have considered it from 30 to 100 nm. They originated when plasma membrane buds inwardly, carrying material from the cell surface and the extracellular space and forming intracellular vesicles. These vesicles then fuse with the early endosome, which moves along the microtubule tracks and as they mature various proteins, including the ESCRT (endosomal sorting complex required for transport) complexes and accessory proteins bind to the surface. The membrane curves inwardly and forms intraluminal vesicles with various types of cargo sorted into them as they form. This structure becomes a multivesicular body (MVB), which traffics to the cell surface along microtubules. The fusion of MVB with plasma membrane leads the release of EXOs (Colombo, M; Raposo, G; Théry, 2014; Schorey & Bhatnagar, 2008). MVs, on the other hand, are larger vesicles (100 - 1000 nm) shed directly from the plasma membrane by exocytosis (Muralidharan-Chari et al., 2008) **(Fig 1.)**. Due to their origin, EVs can carry different types of cargo, including proteins, mRNAs and miRNAs that can be delivered to recipient cells, leading to changes in the cell phenotype and function. EXOs are often enriched with similar molecules of their parent cell and, by that, enriched with a set of proteins involved in antigen presentation, like major histocompatibility complex class I and II (MHC-I and MHC-II) and members of the tetraspanin family (CD9, CD63, CD81 and CD82), various housekeeping, transport and fusion proteins (TSG101, Alix, several annexins and GTPases), that have been currently used as exosomes markers (Brinton, LT; Sloane, HS; Kester, M; Kelly, 2015; Johnstone, 2006; Théry, Zitvogel, & Amigorena, 2002; Trams, Lauter, Norman Salem, & Heine, 1981).

Since EVs have been related with intercellular signaling, understand these vesicle roles, like communication and homeostasis, may highlight some important points of many diseases, such as cancer. EVs are able to modulate tumor microenvironment by interacting with immune cells (Siguo Hao, Moyana, & Xiang, 2007; Schuler et al., 2014). Tumor-promoting inflammation and avoiding immune surveillance have been emerged as hallmarks of cancer (Hanahan & Weinberg, 2011). Tumors have been recognized as organs with high complexity which attain a

unique capability to escape immunosurveillance. Immunoselection and immunosubversion are some ways used by tumor cells to escape from the immune system, which means, respectively, the capability of tumor cells of losing the machinery and sensitivity to immune effectors, and promote active suppression of the immune response (Zitvogel, Tesniere, & Kroemer, 2006). Tumor microenvironment, which is composed mainly by mature and stem cancer cells, endothelial cells, pericytes, cancer-associated fibroblasts and immune inflammatory cells, is extremely complex and still poorly understood. The inflammatory cells may act in opposite ways: tumor-antagonizing and tumor-promoting leukocytes, being the imbalance of this dualistic inflammatory response correlated with the degree of tumor malignancy (Hanahan & Weinberg, 2011). Lymphocytes are fundamental cells in both cellular and humoral immune response, a mark of adaptive immunity and are divided into two major subclasses, B lymphocytes and T lymphocytes (Bours, Swennen, Di Virgilio, Cronstein, & Dagnelie, 2006). T-helper cells can modulate tumor microenvironment secreting cytokines and recruiting additional immune cells, like macrophages. Cytotoxic activity of CD8+ T cells is associated with recognition of antigens expressed by tumor cells and/or some antigen-presenting cells (APC) triggering the effector function necessary for tumor regression. Dendritic Cell (DC) is the most effective APC, which is able to induce a strong immune response *in vivo*. It has been shown ta DC-derived EVs (DEVs) express tumor-associated antigens and have antigen-presentation capability, besides enduing costimulatory molecules (CD80 and CD86), MHC-I, MHC-II and can activate T cell immunity, suggesting they may be a potent vehicle for immunotherapy (Hwang, Shen, & Sprent, 2003; Raposo, 1996; Utsugi-Kobukai, Fujimaki, Hotta, Nakazawa, & Minami, 2003; Zitvogel et al., 1998). In contrast, tumor cells enforce immunosuppressive functions via regulatory cells, mainly Treg cells (Nelson et al., 2001; Poschke, Mougiakakos, & Kiessling, 2011; Wang et al., 2001).

Treg cells are involved in the immune system homeostasis, suppressing an excessive activity of immune cells. Recently, it has been reported the presence of the CD39 enzyme on the surface of human Treg cells. On the other hand, the CD73 enzyme is overexpressed in tumor cells. The synergy between those enzymes presents in Treg cells and cancer cells, promote a more potent immunosuppression profile in the tumor microenvironment. This is due to the fact that these enzymes are involved in purinergic signaling, which are responsible to hydrolyze extracellular ATP to AMP and AMP to adenosine, a well-known immunosuppressive molecule. Briefly, damaged or stressed cells release ATP to the extracellular space. CD39/ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1) presents on infiltrating T lymphocytes converts

that pro-inflammatory ATP, sequentially, into ADP and then to AMP. Subsequently, AMP is converted to adenosine (ADO) by CD73/ecto-5′-nucleotidase (NT5E), overexpressed on cancer cells (Deaglio et al., 2007; Mandapathil et al., 2010; Xu et al., 2013).

As mentioned, EVs are able of interact and modulate lymphocytes, and doing so could carry a cargo of suppressive and stimulate molecules, like tumor-associated antigens (TAA), suggesting that they may play some functions in cancer immunology. In addition, it has been already known that cancer cells highly secrete EVs (Logozzi et al., 2009). Those tumor-derived EVs carry similar molecules of their parent cell, particularly tumor-specific genomic and proteomic signatures and proprieties of tumorigenicity, which leads us to think that their cargo could aggravate the malignant potential of cancer. Additionally, tumor-derived EVs express CD39 and CD73 and lead to production of immunosuppressive ADO, which is another mechanism create by tumor microenvironment promoting tumor escape from the immune system (A. Clayton, Al-Taei, Webber, Mason, & Tabi, 2011). On the other hand, data have shown some EVs contain abundant co-stimulatory molecules and MHC class I and II, which could activate T cell to elicit an effective antitumor immunity (Bu et al., 2015). However, the immunological outcome of EVs is conflicting, concerning whether they may initiate an antitumor immune response or an immunosuppressive response **(Fig 1.)**.

2. Anti-tumoral and pro-tumoral immune response mediated by extracellular vesicles (EVs)

EVs are ubiquitous in the tumor microenvironment, carrying immunosuppressive and immunostimulatory molecules, depending on host and cancer variables such as host immune system, cancer type and stage. Hence, the role of tumor-derived EVs is a controversial matter, with conflicting reports regarding the interaction with surface of immune cells (Muller et al., 2017; Whiteside, 2016). EVs have gained prominence due to its potential as source of tumor antigens for vaccines. The main reports with vesicles bearing immune-activating effects are related to DEVs, which have a well-known antitumor immune response (Hwang, Shen, & Sprent, 2003; Raposo, 1996; Utsugi-Kobukai, Fujimaki, Hotta, Nakazawa, & Minami, 2003; Zitvogel et al., 1998). Some groups have already demonstrated EVs transfer tumor antigens to DC and others APC (Denzer et al., 2000; Morelli et al., 2004).

Zitvogel and colleagues (1998) have shown that tumor peptide-pulsed DEVs from mastocytoma and mammary carcinoma models promote tumor growth delay or complete tumor eradication in mice due to the involvement of $CD8^+$ cells, as well as the presence of MHC I and II and costimulatory molecules, such as CD86 (Zitvogel et al., 1998). Following the same path, Hao et al. (2006) conducted a side-by-side comparison of DEVs and tumor derived EVs (TEVs), demonstrating that both EVs can promote immune stimulatory efficiency and antitumor response; however, DEVs are more immunogenic than TEVs. They also shown that both EVs expressed MHC class I, CD54 and pMHC I complexes, but only DEVs also expressed costimulatory molecules, such as CD40 and CD80, which have been demonstrated as being essential to primary immune responses. Therefore, DEVs strongly stimulate CD8+ T cell proliferation and differentiation leading us to believe DEVs are more immunogenic than TEVs (S Hao, Bai, Yuan, Qureshi, & Xiang, 2006). Bu et al. demonstrated that DEVs, generated by DCs loaded with chaperone-rich cell lysates derived from glioma cell line, exhibited potent anti-tumor activity against mouse glioma and effective T cell immune responses, as cell proliferation and cytotoxic T lymphocyte activity of $CD4^+$ and $CD8^+$ T cells were enhanced, with production of anti-tumor cytokines, such as interleukin-2 (IL-2) and interferon-γ (Bu et al., 2015).

DEVs can also activate the innate immune response. Data have shown DEVs stimulating NK cells via TNF in a dose-dependent manner. The activation and proliferation of NK cells results in tumor rejection (Munich, Sobo-Vujanovic, Buchser, Beer-Stolz, & Vujanovic, 2012). Exosomal expression of NKG2D ligand, IL15Rα and HLA-B-associated transcript-3 had been associated with activation of NK cells (Simhadri et al., 2008; Viaud et al., 2009). Recently, Liu et al. tested a co-delivery of TEVs, derived of C6 cell line, with α-galactosylceramide on a DC-based vaccine. Results showed a potent antitumor response with an iNKT adjuvant, prolonging the survival in 90% of rats treated with α-galactosylceramide + DEX. This treatment also showed increase of $CD3^+CD8^+$ T lymphocytes infiltrated into brain tissues, with high levels of IL-4, IL12p70, IL13 and IFN- γ and low levels of IL-10 in comparison to control group (H. Liu et al., 2017).

Currently, there are also some reports involving TEVs with antitumor activity. TEVs trigger efficient antigen presentation in APCs because they carry tumor antigens (Rao et al., 2016). Related to innate response, it has been already noticed that TEVs from melanoma cells can affect cytokine and chemokine profile in macrophages, activating them and DC (Marton et

al., 2012). Wolfers et al. (2001) have shown EVs immunogenic activity and ability to induce T-cell-dependent antitumor immunity. In melanoma, it has been reported tumor antigens in exosomes, as Mart1, HSP70 and HSP80, besides MHC-I molecules, which are important for immune activation (Wolfers et al., 2001). MHC-II molecules, in EVs derived from B lymphocytes, inducing T cell response were firstly observed by Raposo and colleagues (Raposo et al., 1996). Altieri et al. (2004), have used a vaccine from plasmocytoma-derived exosomes to obtain 80% of tumor reduction when mice were challenged with tumor cells. The authors related this protection with the expansion of specific $CD8^+$ CTL and the presence of several proteins involved in immunity, such as P1A, intracisternal A particle protein and HSP70 (Altieri, 2004). Harshyne et al. (2015) proposed an immunization model by using injections of glioma-derived exosomes (GEX) in mice. They observed those animals that received high doses of GEX had smaller tumors and greater survival than the control group. Immunized animals also showed $CD4^+$ T cells derived from lymphnodes with higher levels of IL-5 (Harshyne et al., 2015). Also, data showed that activation of retinoic acid-inducible gene I (RIG-I) turns TEX into vesicles with an immune-activating and tumor-suppressive phenotype by enhancing NK cell cytotoxicity via BAG6 (Daßler-Plenker et al., 2016).

In addition, some reports have relating mesenchymal stem cells (MSC) and EVs with tumor microenvironment and immunosuppression. For example, these MSC present tropism for tumors and are related with angiogenesis and tumor progression and suppression properties (Djouad et al., 2019; Karnoub et al., 2007; Kéramidas et al., 2013; Khakoo et al., 2006; Zhu et al., 2009). Regarding to EVs, menstrual stem cells-derived EVs have been associated with lower reactive oxygen species (ROS) in metastatic human breast cancer (MD-MB-231 and MCF-7) and human prostate adenocarcinoma (PC3) cells. Lower ROS could affect both NF-κB and VEGF pathways and, consequently, inhibit neovascularization and tumor development. However, the same study showed opposite effect when working with bone marrow stem cells (BMSC)-derived EVs in the same cells lines (Alcayaga-miranda et al., 2016). Lee et al., on the other hand, have shown BMSC-derived EVs are able to suppress angiogenesis by down-regulating VEGF expression in a mouse breast cancer cell line (4T1) (Lee et al., 2013), the same was observed by Pakravan et al., in other breast cancer cells lines (MDA-MB-231, MCF-7 and T47D) (Pakravan, Babashah, Sadeghizadeh, & Mowla, 2017). These findings show that the effect of EVs is dependent on both MSCs origin and tumor type.

As discussed above, EVs carry proteins that are enriched with antigens from the cell origin, including TAA, HSP and costimulatory molecules, which could stimulate the immune

system. Furthermore, EVs also contain tumor-promoting RNAs (mRNA and miRNAs) and proteins (EGFR, VEGF, HSP, KIT, etc) that may mediate suppression by promoting Treg expansion and deliver inhibitory signals to target cells (Al-Nedawi K; Meehan B; Kerbel RS; Allison AC; Rak J., 2009; Atay S; Banskota S; Crow J; Sethi G; Rink L; Godwin AK., 2014; Taylor, 2008). T cells are chronically activated in cancer patients. This leads to greater production of EVs from T cells, which could alter tumor microenvironment. EVs derived from the plasma of head and neck cancer patients induce more or less suppression of T cell proliferation according to the evolution of the disease, in which suppression levels mediated by EVs are lower in healthy donors and patients with reduced disease activity than in high grade patients (Ludwig et al., 2017). Theodoraki et al. (2018) noticed that the cargo of immunoregulatory molecules in $CD3^{+}$ EVs are corelated to the tumor stage, wherein more advanced stage had higher levels of immunosuppressive markers, such as PD-L1, and lower levels of immunosupportive markers, such as OX40 (Theodoraki, Hoffmann, & Whiteside, 2018).

There is a wide variety of publications about the immunosuppression caused by TEVs. For example, impairment in the differentiation of myeloid precursors, like DCs and myeloid-derived suppressor cells (MDSCs) through induction of IL-6 and activation of Stat3 (Chalmin et al., 2010; Y. Liu et al., 2010) and impairment of CTL and innate response induced by Treg and Breg (A. Clayton et al., 2008; Klibi et al., 2009; Yang, Chalasani, Ng, & Robbins, 2012). The main target of direct immunosuppression is the $CD8^{+}$ cytotoxic T cell. In a mice glioma model was observed tumor growth by inhibition of $CD8^{+}$ T cell promoted by glioma-derived EVs (Liu, Wang, & Yuan, 2013). This impairment of cytotoxic cells and APC promoted by TEVs was also noted in PCI-13 and serum patients of head and neck squamous cell carcinoma (Wieckowski et al., 2009). The interaction of TEVs with Treg has been also widely research. TEVs, but not DEVs, induced substantial expansion of $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs, increasing TGF-β production by Treg, anergy and cell death induction of CD8+ T cells (Wieckowsk et al., 2013; Muller et al., 2017). In addition, activated Treg seems to be more responsive to TEVs than others T-cell subsets (Muller et al., 2016).

As shown, TEVs induce immunosuppression by different interactions. Wu et al. (2016) observed that gastric cancer TEVs activated macrophages with a proinflammatory phenotype, stimulating the NF-kB pathway, releasing proinflammatory factors, such TNF-α and IL-6 (Wu et al., 2016). For instance, it has been reported that TEVs (mesothelioma cell lines and blast-derived) down-regulated NKG2D expression on NK and $CD8^{+}$ T cells via TGF-β1 and impaired

IL-15-mediated up-regulation of NKG2D (A. Clayton et al., 2008; Aled Clayton, Mitchell, Court, Mason, & Tabi, 2007; Szczepanski, Szajnik, Welsh, Whiteside, & Boyiadzis, 2011). TGF-β is one of the major cytokines present in TEVs, associated with suppressed NK and T cell proliferation, also promoting tumor development and metastasis via angiogenesis (Bhattacharya et al., 2008; Rong et al., 2016; Szczepanski et al., 2011).

TEVs are also related to induce T cell apoptosis through the receptor-mediated pathway. Signaling of FasL+ EVs via CD95 receptor regulates apoptosis on activated CD8+ T cells (Wieckowski et al., 2009). Interactions with TRAIL/TRAILR, TGF-β/TGF-βR and ADO/A_{2A}R are also related with suppression in the tumor microenvironment. Munich et al. (2012) demonstrated the presence of transmembrane mediators of apoptosis on the surface of DEX, as well as FasL, TRAIL and TNF, which could possibly eliminate antitumoral activity of T cells, at least *in vitro* (Munich et al., 2012). Another results showed the relevance of FasL-mediated apoptosis in tumor evasion, eliminating the efficacy of CTL response by leading lymphocytes to apoptosis (Andreola et al., 2002; Huber et al., 2005). Programmed cell death 1 ligand 1 (PD-L1) plays a major role in suppressing the immune system. It has been observed that the *PD-L1* expression levels are up-regulated in $CD4^+$ and $CD8^+$ Tconv cells, but less in Treg (Muller et al., 2016). TEVs modulate PD-L1 expression of monocytes in leukemia patients by EV-mediated transfer of noncoding RNAs, promoting immune escape via PD-L1 (Haderk et al., 2017). In glioma derived-EVs it was also observed the role of PD-L1 in inhibiting T cell activation, providing another mechanism by glioma to evade immune system (Lubin, Zhang, & Kuo, 2018; Ricklefs et al., 2018).

Considering the ability of EVs to communicate and migrate to distant sites, it is possible to assume they that could be involved in invasion-metastatic cascade. This cascade is a complex process that requires the tumor cells to adapt and grow in a new microenvironment. Briefly, TEVs enhance invasive and migratory potential of recipient cells, and contribute with premestastatic niche formation by carrying a pro-epithelial–mesenchymal transition programme, which includes transforming growth factor beta (TGF-β), hypoxia-inducible factor 1 alpha (HIF1-α), caveolin-1 and β-catenin (Syn, Wang, Sethi, Thiery, & Goh, 2016). Hood et al. injected fluorescently labeled B16-F10 melanoma EVs in mice and observed these EVs have tropism to lymphnodes, initiating a premetastatic niche (Hood et al., 2011). Chen et al. (2018) observed that highly metastatic liver cancer cell line (MHCC97H)-derived EVs improve migration, chemotaxis and invasion of low metastatic hepatocellular carcinoma cells (HCC). The same study observed the MHCC97H-derived EVs induced HCC cells to an epithelial-

mesenchymal transition (EMT, which frequently initiates metastasis) through the MAPK/ERK pathway (Chen et al., 2018). This pathway was also associated with tumor proliferating enhanced by gastric cancer cell-derived EVs (Qu et al., 2009), facilitating invasion and metastasis. Exosomal TGFβ has been also correlated with lymphatic metastasis in a gastric cancer study (Yen, Miaw, Yu, & La, 2017). In addition, multiple exosomal miRNAs (miR-21, miR-423-5p, miR-675, miR-103, miR23a, miR-135b and others) were also identified in promoting tumor metastasis and angiogenesis (Fang et al., 2018; Gong et al., 2018; Park et al., 2010; Umezu et al., 2014; Z. Xu et al., 2018; H. Yang et al., 2018).

3. Adenosinergic system of TEVs promotes immunosuppression

Nucleotide release pathways and purinoreceptors activation are one of the major contributors in the local modulation of inflammation. CD39 and CD73 are the mainly ectoenzymes involved in decreasing the concentration of pro-inflammatory ATP and, consequently, increasing the concentration of anti-inflammatory and immunosuppressive ADO. In the canonical pathway, the enzymatic activity of ectonucleotidases CD39 and CD73 converting extracellular ATP to adenosine acts as negative-feedback mechanism that avoid immune reactions (Stagg & Smyth, 2010). Recent studies have proposed an alternative pathway for the formation of ADO (Malavasi et al., 2008), involving hydrolysis of NAD^+ by CD38 (and by CD157 in a less efficient manner), generating adenosine diphosphate ribose (ADPR) or cyclic adenosine diphosphate ribose (cADPR), which is hydrolyzed by pyrophosphatase/phosphodiesterases 1 (NPP1/CD203a), promoting AMP formation. CD203a can also hydrolyze NAD^+ directly, generating AMP, both ends in canonical pathway, generating immunosuppressive ADO *via* the CD73 enzyme (Horenstein et al., 2013; Malavasi et al., 2008).

The concentration of extracellular ADO is commonly constant in most tissues, but can rapidly increase in hypoxic tissue, in front to an inflammation and has been implicated in the progressive immunosuppression present in cancer microenvironment (Schulte & Fredholm, 2003). As mentioned above, it has been reported the presence of ectonucleotidases, manly CD39, on the surface of Treg cells (Deaglio et al., 2007; Mandapathil et al., 2010). Muller et al. (2016) observed, in the PCI13 cell line and DC, that activated Treg cells have shown the genes coding related to CD25, CD39 and CD73 and adenosine deaminase (CD26) had their expressions levels up-regulated by TEV and DEV, the same wasn't observed in resting Treg

cells (Muller, Mitsuhashi, Simms, Gooding, & Whiteside, 2016). Differences between resting and activated T cell subsets have also been shown by Wieckowski et al. (2009). In her study, it was observed that EVs regulate T cell expansion and TEVs regulate differentially these T cell subsets (Wieckowski et al., 2009).

Recently, it has emerged more evidences detailing the presence of ectonucleotidases in EVs (Clayton, Al-Taei, Webber, Mason, & Tabi, 2011; Morandi et al., 2018; Schuler et al., 2014). This evidence associate the production of ADO by EVs within and outside of the tumor microenvironment and the association of TEVs in pro-tumoral immune responses, as mentioned above. MSC-derived EVs were related to changes in cellular functionality of tumor cells by induction of CD73 activity, promoting alterations in tumor microenvironment and tumor cell proliferation and resistance (Y. Yang et al., 2015). Salimu et al. (2017) observed a dominant effect of prostate-cancer-derived EVs in immunosuppression, Teff and DC function impairment. This suppression was, at least in part, mediated by CD73 expression on DCs triggered by EVs (Salimu, Webber, Gurney, Al-taei, & Clayton, 2017). This immunosuppressive context has also been shown by Clayton et al. (2011), which observed the presence of CD39 and CD73 in EVs derived from different types of sources, like human cancer cell line HT1376 (bladder cancer), CACO2 (colorectal cancer), DU145 and PC3 (prostate cancer) and MCF7 (breast cancer), and in malignant effusions of mesothelioma patients (Clayton et al., 2011). They observed different ATP-hydrolytic activity, CD39 dependent, and 5'AMP-hydrolytic activity, CD73 dependent, relying on cancer cell line. Accordingly, it has been observed that EVs are able to convert ATP in ADO in a sequential manner. This enhancement of ADO levels in tumor microenvironment impairs T cell functions and cytokine release (as IL-2 and IFN-y).

Morandi et al. (2018) have shown an increase of CD38, CD39, CD73, CD203a and CD157 in EVs purified from bone marrow plasma of multiple myeloma (MM) patients compared to controls. They also observed higher consumption of ATP in EVs obtained from patients, consequently, the level of ADO production was also higher, connecting enhancing of ADO production by EVs with an immunosuppressive context (Morandi et al., 2018). Following the same path, another group observed, in MM patients, an elevated expression of CD38 in EVs when compared to healthy controls, indicating those EVs are linked to formation of immunosuppressive ADO (Nielsen et al., 2019). Others also related immunosuppression with the presence of CD38 in EVs in more hematological malignancies, such as leukemia (Caivano et al., 2015; De Luca et al., 2017; Reis et al., 2018). All these reports relate CD38 to

immunosuppression and as a marker of poor prognosis in hematological malignancies. Due to this, CD38 and the non-canonical pathway may be also a promising target for new therapies.

ADO produced by Treg cells can suppress Teff cell function. Also, it is already known that activated T cells can produce EVs to trigger immune suppression. Smyth et al. have shown that Treg cells are able to release EVs presenting CD73 as an important mechanism of immunosuppression (Smyth et al., 2013). Such like has been shown by Shuler et al. by using PCI-13 (head and neck squamous cell carcinoma) and Kasumi-1 (acute myeloid leukaemia) cell lines and plasma from controls and patients with head and neck squamous cell carcinoma (Schuler et al., 2014). The authors also observed overexpression of CD39 and CD73 enzymes in EVs isolated from plasma patients, however mean levels of 5'AMP and ADO production by $CD39^{+}CD73^{+}$ EVs were not different between patients and controls.

4. Conclusion

TEVs carry functional molecular cargo, as proteins, miRNA, mRNA, DNA fragments and oncogenic virus-derived molecules, which could induce malignant transformation and generation of an immunosuppressive environment, reducing the response of Teff cells and triggering the expansion of Treg cells. This potent immunomodulatory effect helps tumor escape from immunosurveillance. However, this molecular and antigenic cargo present on TEVs could also trigger efficient antigen presentation in APCs and, consequently, an anti-tumoral activity. Although it is not completely understood, these opposing results could be explained by the fact that EVs derived from different sources bear specific molecular signatures of their cells of origin, and hence, enclose different molecules, delivering different information into their microenvironments.

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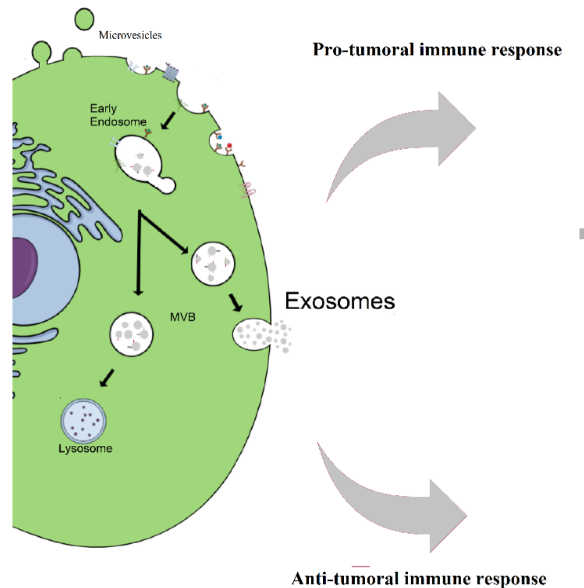
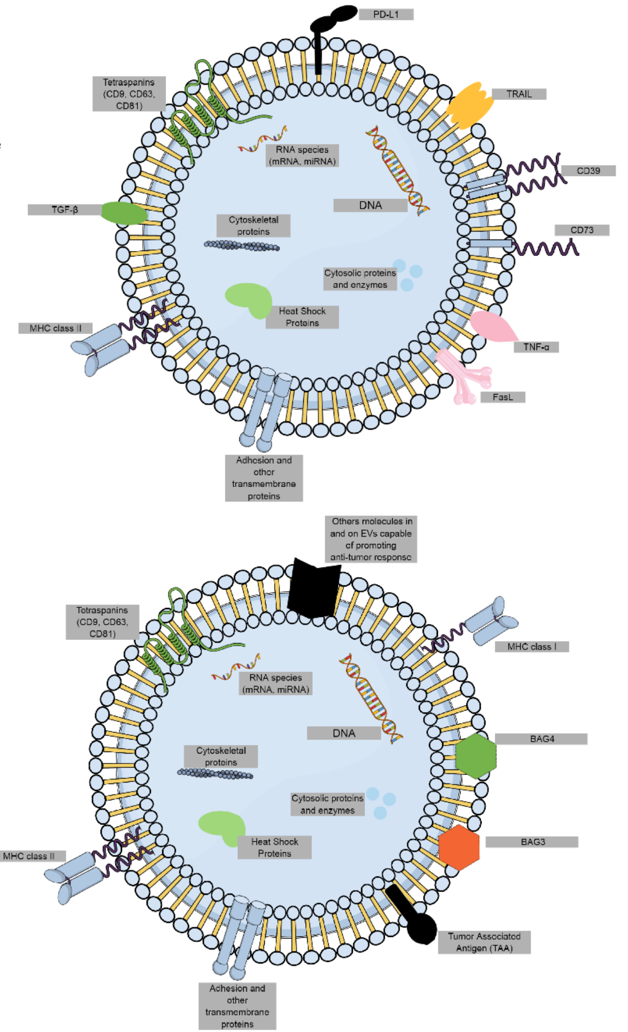
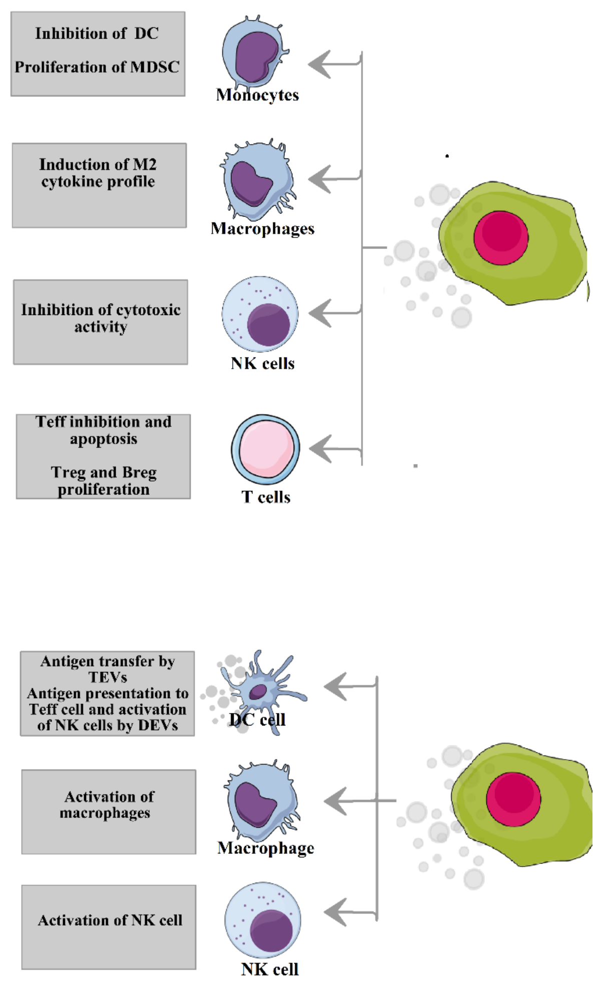


Microvesicles
Early Endosome
MVB
Lysosome
Exosomes
Pro-tumoral immune response
Anti-tumoral immune response



PD-L1
Tetraspanins (CD9, CD63, CD81)
TRAIL
RNA species (mRNA, miRNA)
CD39
DNA
TGF-β
Cytoskeletal proteins
CD73
Cytosolic proteins and enzymes
Heat Shock Proteins
MHC class II
TNF-α
FasL
Adhesion and other transmembrane proteins
Others molecules in and on EVs capable of promoting anti-tumor response
Tetraspanins (CD9, CD63, CD81)
MHC class I
RNA species (mRNA, miRNA)
DNA
BAG4
Cytoskeletal proteins
Cytosolic proteins and enzymes
Heat Shock Proteins
BAG3
MHC class II
Tumor Associated Antigen (TAA)
Adhesion and other transmembrane proteins



Inhibition of DC
Proliferation of MDSC
Monocytes
Induction of M2 cytokine profile
Macrophages
Inhibition of cytotoxic activity
NK cells
Teff inhibition and apoptosis
Treg and Breg proliferation
T cells
Antigen transfer by TEVs
Antigen presentation to Teff cell and activation of NK cells by DEVs
DC cell
Activation of macrophages
Macrophage
Activation of NK cell
NK cell

Figure 1. EVs biogenesis and their role modulating the immune response

Cells are capable of releasing EVs into the cellular environment. EVs biogenesis begins either by direct budding from the plasma membrane, resulting in larger end less complex vesicles, as microvesicles, or by budding into early endosome, which matures into late endosome, also know by multivesicular bodies (MVB). This MVBs formed can fuse with plasma membrane and release exosomes or fuse with the lysosome and undergo degradation by lysosomes. EVS can modulate tumor microenvironment by interacting with immune cells by carrying immunosuppressive and immunostimulatory molecules. The anti-tumoral immune response mediated by EVs can maturate and activate immune cells. NK cells are stimulated by BAG4 and BAG6, DCs are maturated by HSPs, the immune response is activated and mediated by MHC class I and II and tumor-associated antigens (TAA) (a). On the other hand, pro-tumoral immune response mediated by EVs promotes an immunosuppression by inhibition of immune cells and, also, facilitating the activation of regulatory cells. MHC class II can induce immune tolerance, while FasL, TRAIL, PD-L1 and TNF-α induce cell apoptosis, principally of CD8+ cells. Ectonucleotidases are able to produce extracellular adenosine, a potent immunosuppressor, while TGF-β also suppresses immune cells mainly by Treg activity (b).

3.2 CAPÍTULO II

Tumor-Derived Extracellular Vesicles modulate the glioblastoma microenvironment and growth

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Tumor-Derived Extracellular Vesicles modulate the glioblastoma microenvironment and growth

Abstract

Glioblastoma is a deadly type of cancer characterized by a pro-tumoral immune response orchestrated, mainly, by T regulatory cells. Tumor-derived EVs (TEVs) carry a cargo of inhibitory and stimulatory molecules and play an important role in intercellular communication, modulating tumor microenvironment and interacting with lymphocytes. In this study, we propose a method to isolate EVs derived from C6 rat glioma cell lines and evaluate the role of these EVs in tumor progression. TEVs were isolated by ultracentrifugation-protocol and characterized by size, polydispersity and presence of EVs marker, CD9. Also, these EVs can convert ATP in adenosine by CD39 and CD73 enzymes. In vitro studies have shown a decrease of C6 cell viability after treatment with TEVs. For *in vivo* studies, rats with implanted GBM were treated with TEVs by intranasal and intratumoral approaches. EVs decreased tumor size in both approaches and promoted a shift in tumor microenvieroment from pro-tumoral to anti-tumoral. The present results reveal that TEVs are a potential candidate for antiglioma therapy.

Keywords: Glioblastoma, Extracellular Vesicles, Tumor Microenvironment, T regulatory cells

1. Introduction

Glioblastoma (GBM) is the most common and malignant primary glioma of the Central Nervous System (CNS) present in adults, considered the most aggressive (grade IV, referred by World Health Organization) and deadliest type of glioma (Nieto-Sampedro et al., 2011; Robins, Lassman, & Khuntia, 2009; Sathornsumetee et al., 2007). The patient life expectancy is one year despite the standard treatment, which consists of surgical resection and radio/chemotherapy (Behin et al., 2003). The invasiveness, rate of proliferation, immunosuppression and chemotherapy resistance are related with the high recurrence of this cancer (Konopka & Bonni, 2003; Lacroix et al., 2001).

The tumor mass is characterized by a large histological heterogeneity, consisting of tumor cells, endothelial cells, fibroblasts and immune cells, such as macrophages and lymphocytes (Balkwill, 2004; Solinas et al., 2009). In GBM, the presence of an inflammatory

infiltrate has been directly correlated with the degree of tumor malignancy and evidence suggests that the presence of leukocytes in the tumor microenvironment is an indispensable component in the proliferation, migration and tumor survival (Watters, Schartner, & Badie, 2005). Lymphocytes play a central role in both cellular and humoral immune responses, which express specific antigens, receptors and serve as markers of adaptive immunity (Bours et al., 2006). Tumor proliferation is dependent on a complex group of factors, including cytokines, chemokines and nucleosides, like adenosine, which culminate in immunosuppression in tumor microenvironment, orchestrated by, among other cells, Treg cells, which are directly correlated with poor prognosis of patients with GBM (Sayour et al., 2015).

T regulatory lymphocytes are phenotypically classified as $CD4^{+}CD25^{high}FOXP3^{+}$ and control cell renewal in the thymus, regulating lymphocyte expansion, chronic inflammation and autoimmune processes. However, under certain conditions, such as in cancer, Treg cells contribute to an immune suppressed environment by inhibiting the activation of T effector lymphocytes (Teffec) and Natural Killer (NK) and may promote neoplastic growth (Bastid et al., 2013; Borsellino et al., 2016). Treg lymphocytes migrate to the microenvironment of the GBM not only in response chemokines secreted by cells of the immune system, but also by the tumor cells themselves (Jordan et al., 2008). It has been demonstrated that once in the tumor microenvironment, Tregs that overexpress the NTPDase1/CD39 enzyme (Mandapathil et al., 2009), in coordination with GBM cells that overexpress ecto-5'-nucleotidase/CD73 (Bavaresco et al., 2008), produce adenosine by sequential extracellular hydrolysis of ATP. Extracellular adenosine, in turn, is a potent suppressor of immune effector cells such as cytotoxic T lymphocytes (CTL) and NK cells, thus promoting a pro-tumor environment (Xu et al., 2013). Further, Treg lymphocytes also secrete cytokines such as IL-10, TGF-B culminating in immunosuppression in neoplastic tissue, leading to tumor progression (Bours et al., 2006).

In addition to the factors described above, extracellular vesicles (EVs) are also able to modulate tumor microenvironment and interact with lymphocytes (Schuler et al., 2014). Cells secrete EVs, as apoptotic bodies, microvesicles and exosomes, which can vary in size, content and biologic activity. Exosomes (30-150nm) are EVs originated in physio pathological conditions of the endosomal cell compartment (Colombo et al., 2014; Trams et al., 1981). Multivesicular bodies fuse with the membrane releasing exosomes with characteristics from the origin cell. Thus, exosomes may contain different proteins originated from the cytoplasm, endosomes and the plasma membrane of different cells. The functions performed by these EVs are not yet fully known, but some are now being elucidated, for example, participation in

angiogenesis, inflammation and immunomodulation (Aled Clayton, 2012; Théry, Zitvogel, & Amigorena, 2002).

The role of EVs is also controversial, some studies suggest that EVs could inhibit the immune system by adenosine formation via CD39 and CD73 enzymes present in such vesicles and also by stimulating the release of anti-inflammatory cytokines (A. Clayton et al., 2011). Furthermore, when incubated with tumor cell-derived extracellular vesicles (TEVs), Treg lymphocytes secrete higher amounts of immunosuppressive cytokines, being able to inhibit Teffec cells (Szajnik et al., 2010; Wieckowski et al., 2009). In contrast, some other studies suggest that dendritic cell-derived exosomes or from body fluids are able to promote the release of proinflammatory cytokines such as IL-1β, TNF-α and IL-6, leading to an antitumor immune response (Altevogt et al., 2014).

Nevertheless, despite the efforts in recent years, there is still no full understanding of the functions performed by glioblastoma-derived extracellular vesicles (GEVs) (Noerholm et al., 2012). Considering that EVs are enriched with similar molecules of their parent cell and can deliver biological information among cells, these vesicles display a potential importance in onco-immunology field. GEVs may have an important role in the modulation of T lymphocytes in GBM. Therefore, in the present work, a method to isolate EVs derived from C6 rat glioma cell lines were characterized and *in vitro* and *in vivo* studies were performed.

2. **Materials and Methods**

2.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Fungizone®, penicillin/streptomycin, and 0.5% trypsin/EDTA solution were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA). Nucleotides, adenosine (ADO), inosine (INO), hypoxanthine (HYPO), xanthine (XANT), uric acid (UA), methanol, tetrabutylammonium hydroxide, potassium phosphate monobasic and dimethyl sulfoxide (DMSO), 6-N,N-diethyl-D-beta,gamma-dibromomethyleneATP (ARL 67156), Adenosine 5′-(α,β-methylene)diphosphate (APCP) e dipyridamole (DIP) were obtained from Sigma (USA). All other chemicals and solvents used were of analytical or pharmaceutical grade.

2.2 Maintenance of GBM Cell Line

The C6 rat GBM cell line was obtained from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). Cells at passages 5-30 were grown and maintained in DMEM containing antibiotics (0.5 U/mL penicillin/streptomycin) and fungizone, supplemented with 5% (v/v) of Fetal Bovine Serum (FBS). The FBS used for all experiments was microvesicles depleted (MV^-) by two cycles of 3 hours of ultracentrifugation at 105.000x*g* and 4°C. Cells were kept at a temperature of 37 °C, minimum relative humidity of 95% and atmosphere of 5% CO_2 in air.

2.3 Isolation of extracellular vesicles (EVs) enriched with exosomes

Initially, the EVs were prepared using four different protocols based on studies in the literature. Characteristics of each test are described in Table I. After the isolation, the total protein contain was determined through BCA kit (Smith et al., 1985) and EVs size were determined by ZetaSizer Nano ZS equipment. Considering thawing as the most critical parameter for the isolation of smaller EVs, the chosen methodology for further experiments is described as following:

The EVs enriched with exosomes derived from glioma were isolated from the supernatant of GBM C6 cell line. 75 cm³ culture bottles were used to cultivate $7x10^6$ C6 cells for 48 hours with 11 mL of DMEM 5% of FBS (MV^-). After 48 hours, the supernatant was isolated from the culture and centrifuged at 400 x*g* for 6 minutes to remove remained cells and big cells debris. The resultant supernatant was then centrifuged at 2,000 x*g* for 10 minutes at 4°C and 10,000 x*g* for 30 minutes at 4°C, respectively. The supernatant was filtered in a 0.22 µm micro filter and stored at -80 °C. After, the supernatant was thawed at room temperature for approximately 1 hour and centrifuged at 17,000 x *g* for 20 minutes at 4°C. Then, the pooled supernatant was submitted to 2 hours of ultracentrifugation at 105,000 x *g* at 4°C. The resultant pellet from this centrifugation was suspended in sterile PBS and centrifuged again at 105,000 x*g* for 2 more hours at 4°C. The final pellet was suspended in 200 µL of sterile PBS, or lysis buffer for Western blot analysis, and stored at 4°C up to 24 hours. Alternatively, EVs were stored up to 18 days to evaluate their stability at 4°C. The total protein content was determined with BCA kit, in which 10 µL of EV suspension was analyzed in comparison to standard albumin (0.625 - 10 mg/mL). The EVs enriched with exosomes was characterized through the determination of the diameter of the particles and the polydispersity, using the Zetasizer Nano ZS and NanoSight equipments.

2.4 Characterization of EVs derived from GBM cells

Size Characterization

Dynamic Light Scattering (DLS) was performed using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) to determine the mean particle size (Z-average) and polydispersity index (PDI) values of the formulations. The samples were diluted in 1 mL PBS and the parameters such as Z-average and PDI were analyzed at 37ºC (Subra et al., 2010). Nanoparticle tracking analysis (NTA) (using a NanoSight instrument (LM10, NanoSight Ltd., UK) and NTA 2.0 (Analytical Software) was used to analyze the individual particles in the formulations after dilution (5000×) by examining Brownian motion in real time via a CCD camera, and each video clip was captured for over 60 s. Transmission Electron Microscopy (TEM) analysis was performed to evaluate the purity and the diameter of EVs. We proceeded as follow: 10 µL of EVs was dripped onto a grid covered with carbon film (formavar/carbon) and waited to dry. Uranyl 2% was used as contrast. We observed the sample in TEM120Kv (JEM 1200 Exll-JEOL). All measurements were performed in triplicate batches.

EVs metabolism by High Pressure Liquid Chromatography (HPLC)

To analyze purine levels and the action of inhibitors of CD39 (ARL) and CD73 (APCP), as well the action of an inhibitor of ADO uptake (Dipyridamole), EVs were incubated with ATP and HPLC was performed.

Our protocol was based on a previous method described by Voelter et al. (1980) and performed with some adjustments. Briefly, GEVs (8 ug/mL) were pre-incubated for 10 minutes at 37 ºC and the reaction started when the substrate was added to reaction medium in a final concentration of ATP 50 µM. At 30 minutes of incubation, the samples were transferred to ice to stop the reaction. After, samples were centrifuged (14.000 x g for 30 minutes at 4 ºC) and aliquots of 20 µL were applied to a reverse-phase HPLC system (Shimadzu, Japan) using a C_{18} column (Ultra C18, 25 cm x 4,6 mm x 5 µm, Restek-USA). The elution was performed by applying a gradient from 100% solvent A (60 mM of potassium phosphate monobasic and 5 mM of tetrabutylammonium chloride, pH 6.0) and to a 100% solvent B (solvent A + 30% of methanol) over a 30 minute-period. Standards (ATP, ADP, AMP, ADO, INO, HYPO, XANT and UA, 50 µM) were used to evaluate the retention time of each compound separately allowing the identification and quantification. Mixes containing all the standards were used in different

concentrations (1.56 µM to 50 µM) for the calibration curve. Controls were performed to correct the non-enzymatic hydrolysis by adding GEVs after the reactions had been stopped.

Flow Cytometry

To analyze the GEVs' surface proteins and confirm the isolation of EVs, vesicles were coupled to beads and stained with specific antibodies for analysis by flow cytometry (Suárez et al., 2017). Briefly, aldehyde-sulfate latex beads (4 µm, ThermoFisher), able of binding to GEVs, were incubated overnight with GEVs and than were stained with primary rabbit Abs (CD9, CD39 and CD73) or a specific isotype control. GEVs were incubated with secondary Ab specific to primary Ab (FITC-conjugated anti-rabbit secondary Ab, Invitrogen, USA). After, EVs coupled to beads were washed twice and analyzed using BD Accuri™ flow cytometer and the C6 software (BD Biosciences, USA).

2.5 *Assessment of GBM cell viability*

For the 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, 2 x 10^3 C6 glioma cells were seeded in 96-well plates and allowed to grow until semi-confluence. Cells were treated with 1.0; 2.0; 4.0; 8.0; 16.0 and 32.0 µg/mL of EVs for 96 h. PBS was used as vehicle. At the end of the treatment, MTS was added to each well and the plate was incubated for 2 hours at 37°C. After incubation, the plate was read at 490 nm in SpectraMax M5.

2.6 *Isolation of mesenteric lymphocytes and flow cytometry*

Mesenteric lymph nodes were isolated from adult Wistar rats (8-9 weeks old, 220-300 g) and dissociated through a mesh in 10% RPMI medium (Scherer et al., 2012). One million cells were centrifuged at 400 xg for 6 minutes twice, seeded in a 6-well plate and treated with 8.0 µg/mL EVs for 48 hours. CD39 and CD73 expression in total lymphocytes and in $CD4^+$ and $CD8^+$ lymphocytes was evaluated by flow cytometry using rabbit ant-rat CD39 (1:400; http://ectonucleotidases-ab.com) and CD73 (1:200; http://ectonucleotidases-ab.com) primary antibodies (Ab), FITC-conjugated anti-rabbit secondary Ab (1:100, Invitrogen, USA) and rabbit anti-rat CD25-PE, CD8-PerCP and CD4-APC conjugated Ab (all from BD Bioscience, USA). Briefly, cells were incubated with primary Ab against CD39 and CD73 for 30 minutes and washed twice. After, cells were incubated with secondary Ab specific to primary Ab and

with lymphocytes conjugated Ab for 30 minutes in dark. After incubation period, cells were washed twice and analyzed using BD Accuri™ flow cytometer and the C6 software (BD Biosciences, USA). There were two different staining sets: CD39-FITC/CD25-PE/CD8-PerCP/CD4-APC and CD73-FITC/CD25-PE/CD8-PerCP/CD4-APC. A secondary antibody or isotype control was used as a non-specific binding control.

2.7 *In vivo GBM model*

GBM implantation was performed as described previously (Figueiro et al., 2015) Briefly, C6 rat glioma cells at approximately 80% of confluence were trypsinized and a total of $3X10^5$ cells in 3 µL were injected at a depth of 6.0 mm into the right striatum (coordinates relative to Bregma: 0.5 mm posterior and 3.0 mm lateral), using a Hamilton microsyringe coupled with an infusion pump (1 µL/min) of anesthetized adult male *Wistar* rats (8-9 weeks old, 220-300 g) by intraperitoneal (i.p.) administration of ketamine/xylazine.

For tumor size quantification, four hematoxylin and eosin (HE) sections (3 mm each) from each animal were analyzed and images were captured with a digital camera connected to a microscope (Nikon Eclipse TE300). The tumor area (mm^2) was determined using software ImageJ and the total volume (mm^3) was established by the multiplication of the slice sections. For tumor microenvironment analysis, tumors were dissociated by enzymatic digestion with collagenase IV. After the dissociation, cells were harvested twice by centrifugation in PBS at 400 xg for 6 minutes, counted and analyzed by flow cytometry using CD4-APC, CD25-PE, CD39-FITC, FOXP3-FITC.

Animal treatments

I- Immunized Group: The animals received intranasal administration (IN) of 40 µg GEVs divided in two doses (20 µg each) or PBS (control group), administered 10 and 5 days before C6 GBM cells implantation. Fourteen days after implantation, the rats were decapitated and the entire brain was removed.

Groups:

1) Animals pretreated IN with vehicle (PBS);
2) Animals pretreated IN with GEVs (40 µg).

II- Coinjection Group: The animals received C6 GBM cells resuspended in DMEM without FBS. Alternatively, cells were coinjected with dispersed GEVs 1,5 µg in DMEM without FBS. Fourteen days after implantation, the rats were decapitated and the entire brain was removed.

Groups:

1) Animals with GBM implantation in DMEM solution;
2) Animals with GBM implantation in GEVs solution (1,5 µg).

All of the procedures used in the present study followed the "Principles of Laboratory Animal Care" from the National Institutes of Health (NIH) and were approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul (Protocol # 33505).

2.8 Statistical analysis

The data were analyzed for statistical significance by Student's t-test or one-way analysis of variance (ANOVA) followed by a post-hoc test for multiple comparisons (Tukey test), using GraphPad Prism software®. The data are expressed as the mean ± S.D. Differences were considered significant at $p < 0.05$.

3. **Results**

3.1 Determination of isolation protocol

Firstly, we describe the variations tested to determine the best isolation protocol using differential centrifugation. Initially, we searched for previous procedures reported in literature to determine the best experimental conditions by using differential centrifugation as the major procedure.

Four unexplored experimental conditions were tested to determine the best protocol for isolation, considering EVs size and protein content (Table I). Average size was determined by ZetaSizer Nano ZS equipment and protein amount were quantified with BCA-kit after 24 h from isolation. The main differences among the tests were: (1) filtration (0.22 µm) after overnight thawing at 4°C; (2) additional centrifugation (17,000xg, 30 min, 4°C) after overnight thawing at 4°; (3) filtration (0,22 µm) and additional centrifugation (17,000xg, 30 min, 4°C) after 1 h thawing at 25 °C; (4) thawing at 25°C for 1 h. In test 1 (Table II), we obtained higher results for protein concentration (1.395 µg/µL); however, the percentage of big vesicles, like microvesicles, was also higher (277.5 nm). In test 2, we also obtained a bigger particles size

(208 nm) than test 3 (145 nm) and test 4 (159 nm) both with a reliable protein content. Exosomes are described as particles with 30-150 nm, so a smaller average size indicates a higher presence of these particles beyond the presence of other EVs and isolated of EVs with mean diameters around 150 nm tend to be enriched in exosomes. Thus, isolation 4 was chosen as the best condition for our aim and was used in the following experiments.

After the results showed in Table II, we figured that the filtration after the thawing was not necessary and it did not substantially interfere with the size of isolated EVs and leading to lower yields. Therefore, the faster thawing approach and the additional centrifugation after the thawing were decisive to obtain the desired EVs (complete isolation technique is described in M&M section).

Finally, EVs isolated through the chosen protocol) were submitted to three protein analysis: Lowry, Bradford Adapted and BCA-kit. The protein concentration determined by Lowry and Bradford assays was around the inferior limit of quantification, different from the BCA assay, where the protein concentration is around the middle of the standard curve, besides the greatest correlation coefficient (data not shown). Thus, BCA method is the best technique to quantify EVs protein. Therefore, we have chosen this technique to determine protein concentrations in the following experiments.

3.2 Size characterization of EVs derived from GBM cells

After determining the isolation and protein quantification protocols, we decided to compare the size results obtained from two used equipment to this finality, ZetaSizer and Nanosight, up to 24 hours after isolation. Analyzes from Nanosight assay demonstrate a polimodal curve, separating different populations of different particle sizes and showing the diversity of the EVs sizes (FIG 1A). ZetaSizer demonstrated a monomodal curve, which combine all different particles present in the sample (FIG 1C). In Nanosight equipment, sensitivity was able to separate subpopulations of EVs, leading to more reliable results with minor variations among different analysis (Table III). Still, Nanosight capability to provide approximate submicron particle counts is an advantage of the equipment over ZetaSizer, indicating that Nanosight may be a better choice to scattered EVs isolate. The Nanosight results demonstrated a presence of small subpopulations with size greater than 200 nm. These particles may be either the result of EVs aggregation or larger isolated EVs (microvesicles, for example). Table III also shows the reproducibility and polydispersity through indices D10, D50 and D90, which indicates the percentage of particles undersize this percentile in size distribution curve.

The particles size distributions obtained in three different experiments are reproducible among themselves. As shown, the size of our isolate is around 50% smaller than 150 nm. EXOs size range 30-150 nm, so our samples are not composed only by EXOs, but also presented some larger particles such as microvesicles. Confirming this, as shown in the TEM images (FIG 1D), GEVs were successfully isolated by our method of isolation, showing the classical "cup-shaped", "doughnut-like" morphology and heterogeneous size.

The above mentioned experiments were performed up to 24 hours after the EVs isolation. To determine the stability of the preparation, the EVs size was also determined up to 4 and 18 days after the EVs isolation by ZetaSizer Nano ZS. Figure 1C shows the profile of EVs at 1, 4 and 18 days. After 18 days of EVs isolation there was a slightly increase in the subpopulations with bigger size than the EVs stored at 4°C for 1 and 4 days.

3.3 GEVs express CD39 and CD73 enzymes

To confirm that the isolated structures are EVs, we assayed the presence of CD9, a molecule frequently used as an EVs marker, by Flow Cytometry. We also investigated the presence of CD39 and CD73 enzymes on EVs by these techniques and then analyzed the ATP metabolism of this vesicles by HPLC. Through flow cytometry, we could observe that GEVs express all the markers tested, being CD9 the most abundant (61,9 ± 6,12 %), followed by CD73 (46,5 ± 5,45 %) and CD39 (23,6 ± 7,26 %) (Fig 2A). The same was observed by western blotting analysis, confirming also the presence of HSP70 (FIG 2C). For further characterization, 8.0 μg/mL of GEVs were incubated with ATP 50 μM and treated with inhibitors of CD39 and CD73 enzymes (ARL and APCP, respectively), as well as with an ADO uptake inhibitor (dipyridamole/DIP). Inhibitors of CD39 and CD73 enzymes reduced ADO formation (52,1 % and 57,8%, respectively), confirming, again, that adenosine formation via sequential action of CD39 and CD73 enzymes on GEVs (FIG 2B). ADO transporter blockade by DIP did not affect the extracellular concentration of this nucleoside since the ADO levels, when apply DIP, resembles control values.

3.4 Effect of GEVs treatment in a rat glioma cell line

To investigate the direct interaction of GEVs with C6 glioma cell line, MTS assay was performed. Concentrations between 1 - 32 μg/mL of EVs were used in cells treated for 96 h. Analysis of MTS assay showed that 16 and 32 μg/mL of EV were able to cause a significant

reduction in cell viability when compared with the PBS group (30% and 24% decrease, respectively) (FIG 3). Treatment with the vehicle PBS does not affect cell viability.

3.5 GEVs did not affect CD39 and CD73 expression on peripheral T lymphocytes

Considering EVs can interfere in modulation of immune cells, we tested the role of GEVs on peripheral lymphocytes. CD39 and CD73 expression were measured on mesenteric lymphocytes of Wistar rats after treatment with 8 µg/mL of GEVs for 48 hours. The expression of these enzymes was evaluated by flow cytometry on total T lymphocytes and CD4+ and CD8+ subsets. Enzymes expression was not affected by the treatment in any tested lymphocyte subset (FIG 4).

3.6 GEVs suppress *in vivo* glioblastoma growth and modulate the lymphocytes in the tumor microenvironment

To assess the antitumoral activity of GEVs *in vivo*, we established two rat glioma models. Firstly, in the coinjection model, 1,5 µg GEVs were coinjected with C6 glioma cells and 14 days after tumor growth the brain was removed. Secondly, we used an intranasal drug delivery, a promising and noninvasive pathway to deliver several therapies for treatment of brain disease, like GBM. Because of that, in our model of immunization, we pretreated twice the animals with GEVs 40 µg via intranasal administration, as described in materials and methods, and after 14 days of tumor growth the brain was removed. Treatment of the animals bearing GBM with GEVs coinjection resulted in 45% reduction of tumor volume in comparison with control group (from 221 ± 65,1 mm^3 to 121± 40,6 mm^3). Corroborating with the coinjection model, intranasal administration of GEVs also resulted in 60% reduction of tumor volume in comparison with the control group (from 173 ± 91,8 mm^3 to 69 ± 20,2 mm^3). Seeking to understand the mechanism behind this reduction, we analyzed the presence of $CD4^+FOXP3^-$ and $CD4^+FOXP3^+$ cells in tumor microenvironment. The coinjection group showed a significant reduction of $CD4^+FOXP3^-$ and $CD4^+$ $FOXP3^+$ cells, however we didn’t observe the same in IN group, which has shown no difference between groups. This could be related to the fact that in coinjection group GEVs are injected right in the moment of tumor bearing and hence the vesicles act more locally, promoting a significant shift in tumor microenvironment. In the immunization group, there was no difference between groups and this may be due to the possibility of the vesicles acting more systemically, because the groups receive the treatment

before tumor bearing. These data indicate that GEVs may be a promising strategy to control in vivo GBM growth.

6. Discussion

GBM is the deadliest tumor of CNS and remains one of the main challenges in oncology field due to invasiveness, proliferation, vast intratumoral heterogeneity and poor prognosis. Tumor microenvironment and its interactions with the immune system are crucial in the progression of the disease. Further, EVs play an important role in intercellular communication and are capable to modulate the tumor microenvironment and interact with the host immune system; however, their role in tumor progression still a contentious issue. In this context, the present study proposed a protocol to isolate EVs in a proper quality and quantity and demonstrate that EVs derived from C6 rat GBM cell line are able to decrease *in vitro* and *in vivo* glioblastoma growth, promoting a shift from a pro-tumoral to an anti-tumoral microenvironment.

Until now, there are plenty of different methods for isolation, detection and characterization of EVs, turning difficult the interstudies comparison. This is because EVs are nanometric in size and, despite of the method, difficult to isolate and detect. Like most researches, in this work we used an ultracentrifugation-based protocol to isolate EVs from the supernatant of rat GBM cell culture due to the type of sample and lower cost of the technique. Among some variations tested in the technique, time of thawing was one of the decisive step, when a fast thaw, gave to us smaller particles with reasonable protein concentration (Table I and Table II). Analysis of EVs falls into some main categories, as size, number and content. Although TEM is the standard imaging method for observing nanosized particles, as EVs, DSL and NTA are the most performed by other researches (Chuo, Chien, & Lai, 2018; Vestad et al., 2017). Here, we performed a comparison between Nanosight and ZetaSizer techniques. Nanosight are able to detect subpopulations of EVs and provide EVs concentration, leading to more reliable results with minor variations among different analysis, indicating this equipment may be a better choice to scattered EVs isolate, as determined by Zhang at al. (2015). ZetaSizer, on the other hand, provides a combination of all particles presents on isolate and, therefore, afford a greater variability between different analyses since the subpopulations present in the sample may vary among the isolations. TEM is more useful to verify the morphology and presence of intact EVs. We also evaluated EVs stability through the days and observed that

these vesicles remain stable at least for 18 days at 4 ºC. There are some data relating how biofluid storage methods can influence EVs composition, yield and function (Ge et al., 2014; Welch et al., 2017; Zhou et al., 2006). With regard to EVs isolated from cell culture supernatants, there are only few knowledge about storage and stability. The International Society for Extracellular Vesicles (ISEV) recommends that EVs should be stored at -80 ºC, in PBS (Witwer et al., 2017). Capricor Therapeutics, in a patent of processes for producing stable EVs formulations, demonstrates EVs size and concentration remain stable after one week of storage at 4 ºC, -20 ºC and -80 ºC. However, miRNA levels decrease during this period at 4º and -20ºC, but not at -80ºC (Kreke et al., 2015). Generally, those reports agree that the most suitable form of vesicle storage is at -80 °C, despite still a lack of studies regarding EVs stability after freeze-thaw cycles. Therefore, considering the loss of EVs concentration due to freeze-thaw cycles and, in our stability experiment, the increase of size was very small, for short time storage, 4ºC can be useful at least for cell culture supernatant.

Because of their origin, EVs derived from different cell types are enriched by membrane proteins of their source cells. Some protein markers are present in literature to characterize exosomes in mixed EVs populations, like CD9. After determine the worth method to isolate and characterize vesicles for posteriors assays, we demonstrated that our isolates are enriched by EXOs due to the presence of classical protein markers, like CD9. According to ExoCarta, this marker are the most identified in EXOs (Keerthikumar et al., 2016). We also observed this vesicles are able to convert ATP in adenosine (FIG 2B) and confirm the presence of CD39 and CD73 by flow cytometry (FIG 2A), leading us to believe that GEVs participate in extracellular adenosine production, as has been shown in other types of cancer (A. Clayton et al., 2011; Schuler et al., 2014). Adenosine accumulated in tumor microenvironment promotes tumor growth, angiogenesis and inhibit antitumor immune response by suppressing Teff cell function and up regulating Treg cell suppression functions (Deaglio et al., 2007; Mandapathil et al., 2010; Ohta et al., 2006). Hence, this accumulation of ADO may lead to a protumoral role of GEVs. Interestingly, our *in vitro* and *in vivo* data showed the opposite. EVs are able to interact and modulate lymphocytes, carry suppressive and stimulate molecules of their parent cell, particularly tumor-associated antigens (TAA), tumor-specific genomic, proteomic signatures and proprieties of tumorigenicity, which lead us to think that resulting cargo may play complex and yet non-well establish functions in cancer immunology, depending on cancer cell type and, therefore, cargo of EVs interacting in a very defined context (Logozzi et al., 2009). Also, it's important highlight the brain has an immune-specialized nature, which even in absence of

tumor, has immunological preference in secretion of immunosuppressive molecules, as TGF- and induction of Th2 response (Kiefer et al., 1994). Previous studies have demonstrated some controversial role of EVs, concerning whether they may initiate an antitumor immune response or an immunosuppressive response (Chalmin et al., 2010; Daßler-Plenker et al., 2016; Liu et al., 2010).

As mentioned, EVs modulates lymphocytes, however, in our *in vitro* experiment, we did not see any alteration in peripheral T lymphocytes regarding purinergic enzymes after GEVs exposition, showing any involvement or the need of multiple factors absent in an in vitro experiment. Regarding our *in vivo* results, our study demonstrates an antitumoral activity of GEVs. This suggests that there may be other exosomally associated factors that contribute to initiate an antitumor immune response. In cancer immunotherapy, EVs application originally was based on the observation that dendritic cells (DC)- derived EVs, riches in CD9, CD81 and MHC I and II, can replace DC in immune response induction (Delcayre, Shu, & Pecq, 2005). TEVs, although the well-known immunosuppressive activity through inhibition of Teff activity, stronger suppressive activity of Treg cells and impaired activity of NK cells (Clayton, Mitchell et al., 2007; Zhang et al., 2007), supports that immune response induction EVs heat shock proteins promote NK activation, tumor cell lysis by release of granzyme B and induce a tumor antigen- specific cytotoxic T cell response (Dai et al., 2005; Khalil et al., 2011).

Also, TEVs can provide a source of shared tumor Ags. Our model show a significant reduction of the tumor after treatment with GEVs and, to support this data, a shift in the tumor microenvironment from pro-tumoral to antitumoral, with an important reduction of immunosuppressive Treg cells after the treatment. The exact mechanism behind it is not well understand, however, we believe the GEVs carry antigenic material and major histocompatibility complex peptide complexes, which are required in the antigen-presenting process of immune responses, promoting a strong activity of Teff cells and suppressing Treg cells. Some reports also related the immunogenic role of TEVs associated with release of proinflammatory cytokines, as IL2 and IL18 (Dai et al., 2005; Yang et al., 2007) and activation of DC (Chen et al., 2011). TEVs also overexpress membrane-bound HSP70 which can activate $CD8^+$ T cells and NK cells (Xie et al., 2010).

As we can observe in Figure 5, there are an important reduction of Treg ($CD4^+FOXP3^+$) after GEVs coinjection (Fig 5C), this is the reason, at least in part, of reduction of tumor size after this treatment. At the same time, $CD4^+FOXP3^-$ also reduces (Fig 5E). These cells can be T naives, their reduction in tumor microenvironment and its decrease may affect the formation

of induced Tregs in this site (Silva et al., 2016). This also corroborates with the decrease in tumor size after GEVs coinjection treatment. On the other hand, we can't observe this clear shift in tumor microenvironment in GEVs intranasal treatment. In this model, the intervention proposed is an immunization, like a vaccine. The decrease in tumor size in the absence of change in $CD4^{+}FOXP3^{+}$ in tumor microenvironment can be related with the type of response obtained in this model. The immunization can elicit CD8+ memory T cells and promote a protective role.

Although there are studies relating GEVs with immunosuppression of the microenvironment and consequent tumor progression (Domenis et al., 2017; Hellwinkel et al., 2016; Iorgulescu et al., 2016), few data are available showing antitumoral effect of GEVs (Bronisz et al., 2014; Katakowski et al., 2013; Muller et al., 2015). To the best of our knowledge, this is the first study demonstrating the EVs derived from C6 rat glioma cell line suppressing *in vivo* glioblastoma growth and modulating the tumor microenvironment. It become clearly that GEVs impact immune cells, but the nature of those interactions and the molecular drivers remain unknown. Therefore, our study suggests that GEVs may be an efficient tumor vaccine.

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Compliance with ethical standards: All of the procedures used in the present study followed the "Principles of Laboratory Animal Care" from the National Institutes of Health (NIH) and were approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul (Protocol #33505).

Conflict of interest: The authors declare that they have no conflict of interest.

Table I. Experimental conditions tested to determine EV isolation protocol through differential centrifugation

Test / Experimental condition	1	2	3	4
Number of cells in culture (7 x 10^6 cells/75 cm³ bottle)	✓	✓	✓	✓
Centrifugation 1 (400g, 6min, 25 ºC)	✓	✓	✓	✓
Centrifugation 2 (2000g, 10min, 4°C)	✓	✓	✓	✓
Centrifugation 3 (10000g, 30min, 4°C)	✓	✓	✓	✓
Filtration (0.22 µm)	✓	✓	✓	✓
Freeze (-80°C)	✓	✓	✓	✓
Thaw	Overnight at 4-8°C	Overnight at 4-8°C	1 hour at room temperature	1 hour at room temperature
Filtration (0.22 µm)	✓	-	✓	-
Centrifugation 4 (17000g, 30min, 4°C)	-	✓	✓	-
Centrifugation 5 (105000g, 2h, 4°C)	✓	✓	✓	✓
Centrifugation 6 (105000g, 2h, 4°C)	✓	✓	✓	✓

Table I. Experimental conditions tested to determine EV isolation protocol through differential centrifugation. We performed 4 tests to determine the best condition to isolate EV from C6

glioma cells supernatant. (✓) represent the presence of the step in the test and (**-**) represent the absence of the step in the test.

Table II. Average size and protein quantification of different protocols

Test	Average size (nm ± S.D.)	Protein quantification (µg/µL)
1	277,5	1,395
2	208,0 ± 16,1	0,930
3	145,1 ± 2,2	0,953
4	159,3 ± 4,3	1,227

Table II. Average size and protein quantification of protocols tests. The tests 1-4 were performed as described in Table I. After the EV isolation, they were submitted to ZetaSizer Nano ZS to determinate particles size and BCA assay to determinate protein concentration.

Table III. Data from Nanosight analysis

Experiment	Concentration (particles/mL)	Average size (nm)	SD (nm)	D10 (nm)	D50 (nm)	D90 (nm)
1	7,23 x 10^8 ± 0,22	171,15 ± 2,9	41,65 ± 1,9	113,15 ± 1,06	160,1 ± 0,07	217,25 ± 15,6

Table III. Data from Nanosight analyzes. After the determination of the isolation protocol, three samples were submitted to Nanosight analysis in triplicate. SD: standard deviation calculated by the NTA. The results of size and polidispersity (D10 and D90, that represents the percentile of particles undersize 10 and 90) of each sample are demonstrated as experiment 1-3.

Figure 1. Size characterization and stability of EVs

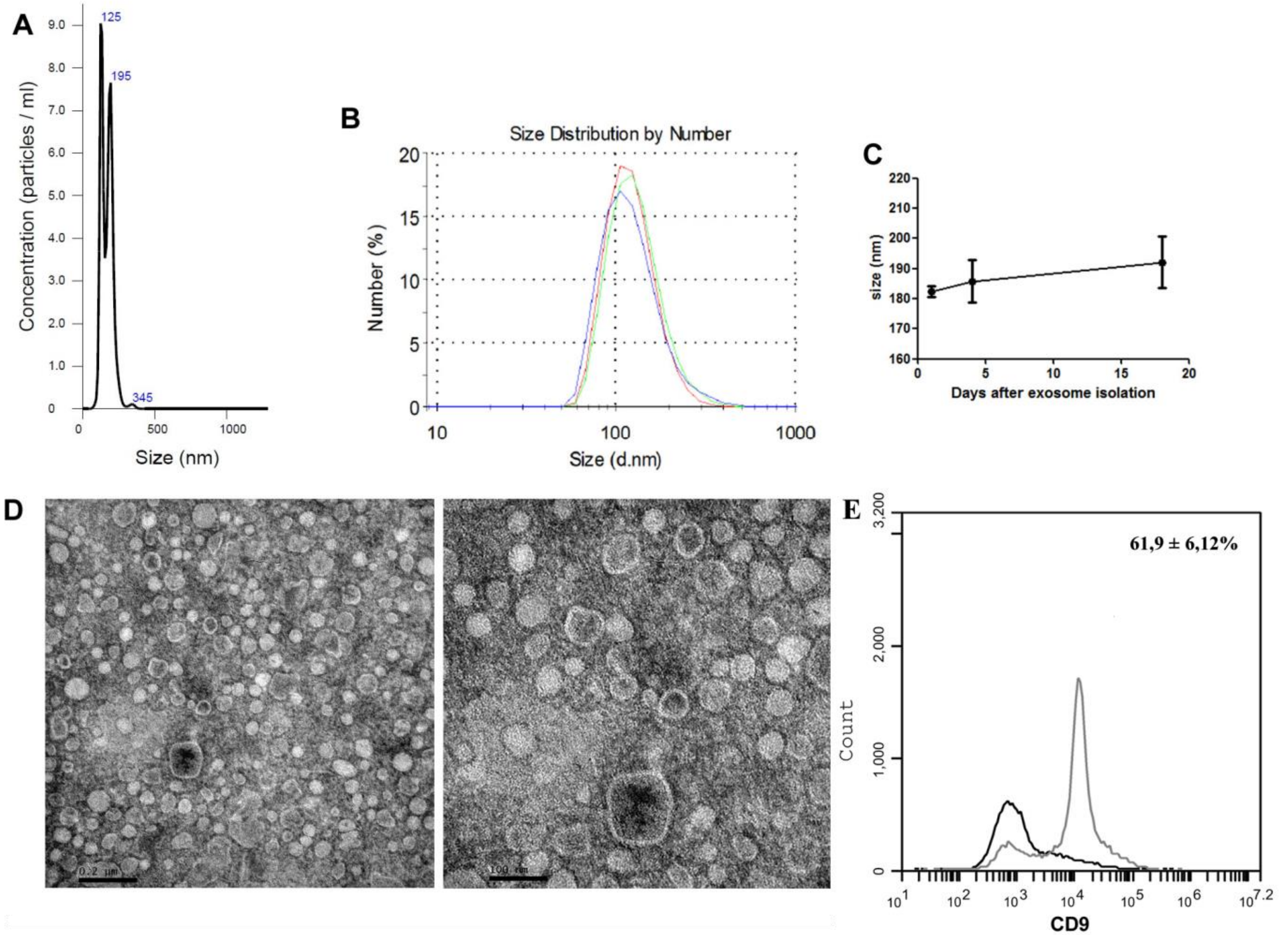

Figure 1. GEVs size and stability. The EVs were isolated as described in MM. After 24 hours of EVs isolation, the samples were submitted to two different size analyses: ZetaSizer and Nanosight equipments. (A) Representative graph (triplicate) from one sample analysis in Nanosight; (B) Representative graph (triplicate) from one sample analysis in ZetaSizer (175,2 ± 6,14 nm); (C) Size measurement in ZetaSizer up to 1, 4 and 18 days after EVs isolation; (D) Transmission electron microscopy of EVs. Magnifications 100K and 200K, bars represents 0.2 um and 100 nm, respectively; (E) EVs expresses CD9, the classical marker of EXOs. Values are presented as mean ± S.D.

Figure 2. Characterization of GEVs

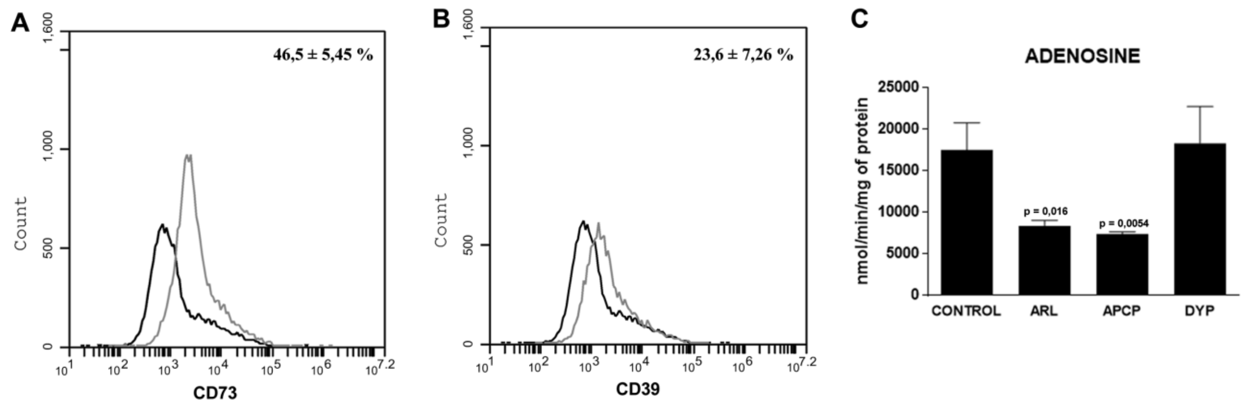

Figure 2. GEVs express CD73 and CD39 enzymes

Expression of CD73 and CD39 by flow cytometry (n = 3). Concentrations of ADO are reduced after treatment with inhibitors of CD39 and CD73 enzymes (ARL and APCP, respectively). The addition of Dipyridamole, an inhibitor of ADO uptake, did not change ADO levels. The results are expressed as mean ± S.D.

Figure 3. MTS assay

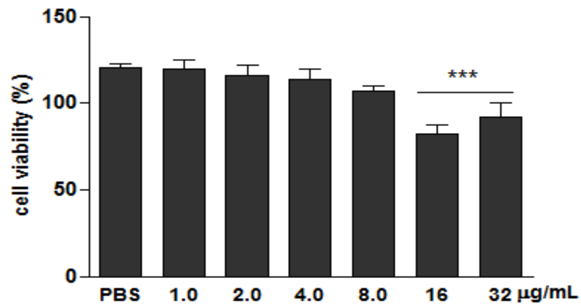

Figure 3. GEVs reduces the GBM cell viability *in vitro*

C6 GBM cells were treated with different concentrations of EVs for 96 h (n = 3) and cell viability was assessed by MTS assay. Percentage of viable cells was significantly reduced using 16 and 32 μg/mL of EVs. The results are expressed as mean ± S.D.

Figure 4. GEVs Incubation with mesenteric lymphocytes

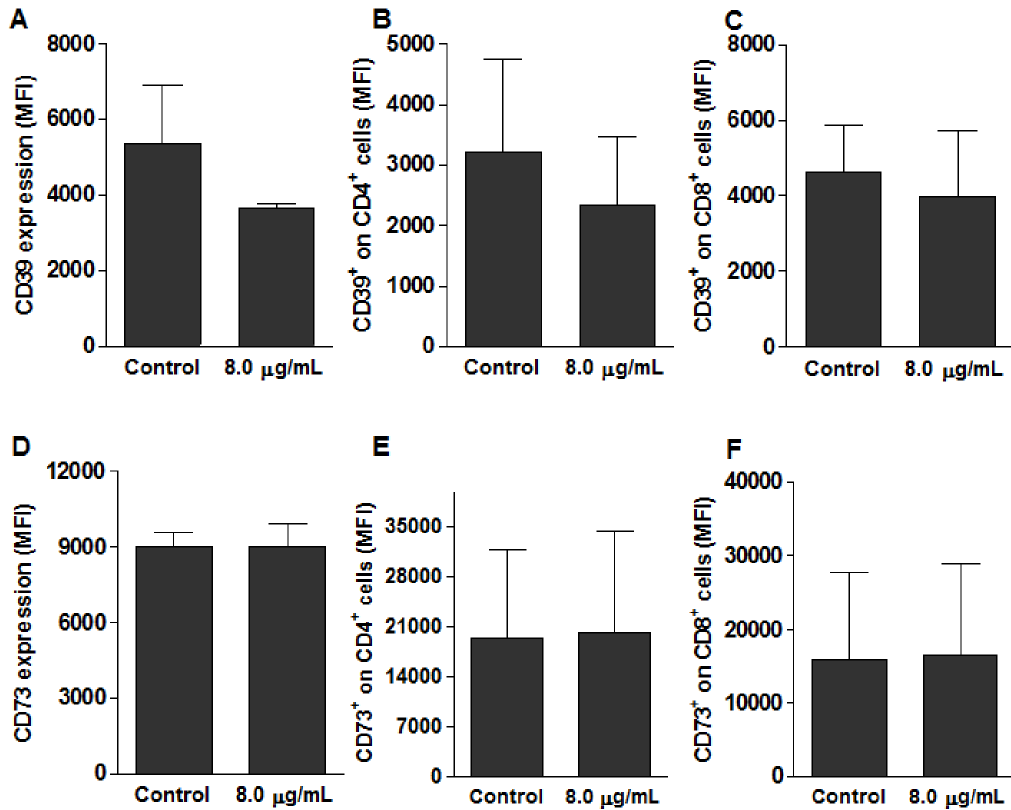

Figure 4. GEVs incubation with mesenteric lymphocytes did not affect the expression of CD39 and CD73 enzymes.

Mesenteric lymphocytes were isolated from Wistar rats and treated with 8 μg/mL GEVs for 48h (n = 4). Expression of the CD39 enzyme in total lymphocytes (A), in CD4 T lymphocytes (B) and CD8 T lymphocytes (C). Expression of the CD73 enzyme in total lymphocytes (D), T helper (E, CD4 +) and T cytotoxic (F, CD8 +). The results are represented by the mean fluorescence intensity (MFI) and are expressed as mean ± S.D.

Figure 5. *In vivo* model

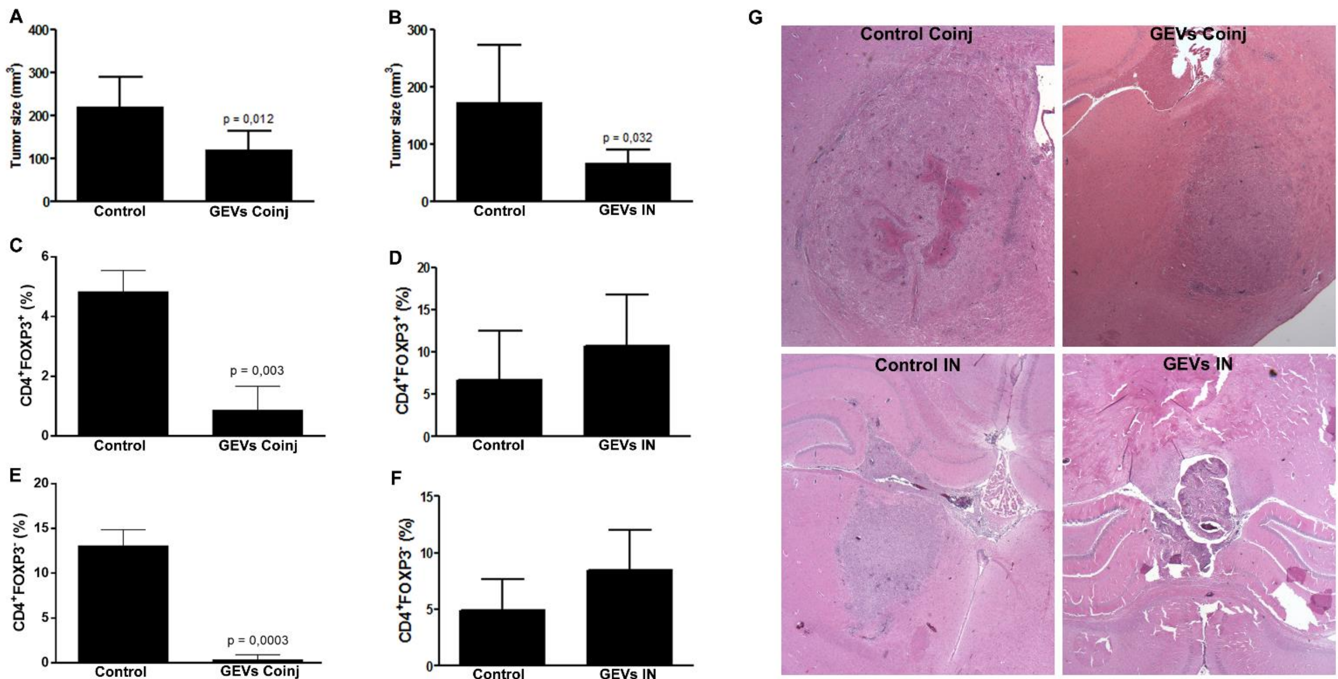

Figure 5. *GEVs reduced the tumor size in the in vivo models*

The in vivo GBM models was performed by injecting C6 GBM cells into the striatum by stereotactic surgery of adult Wistar rats. Two models were performed (a) coinjection model (control n = 7/GEVs n = 6) and (b) immunization model (n = 6). The co-injection of EVs and GBM cells reduced the tumor size from 221 ± 65,1 mm³ to 121± 40,6 mm³ in comparison to GBM group (a). Intranasal administration of GEVs reduced from 173 ± 91,8 mm³ to 69 ± 20,2 mm³ (b). Tumor microenvironment in coinjection model (n = 3) reduce Teff and Treg cells (c and e), in immunization model (n = 4) showed no difference (d and f). The images are in 20X magnification. The results are expressed as mean ± SD.

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PARTE III

4 DISCUSSÃO

Gliomas malignos representam um dos maiores desafios no manejo dos pacientes com câncer em todo o mundo. Os tumores cerebrais primários são uma das neoplasias mais refratárias e sua forma mais agressiva, o GBM, também é o subtipo mais comum e letal. Apesar de recentes e notáveis descobertas feitas em oncologia, usando técnicas de neuroimagem na ressecção cirúrgica, juntamente com quimioterapia e radioterapia, a sobrevida dos pacientes atinge em média somente 15 meses a partir do momento do diagnóstico (Stupp et al., 2009).

O prognóstico desfavorável dessa doença está associado ao potencial invasivo e proliferativo que esse tumor possui, além da vasta heterogeneidade intratumoral presente. O microambiente tumoral e sua interação com o sistema imune são cruciais para a progressão da doença. Nesse aspecto, vesículas extracelulares derivadas de tumores (VETs) desempenham um importante papel na comunicação intercelular, capazes de modular o microambiente tumoral e interagir com o sistema imune; contudo, seu papel pró-tumoral ou antitumoral na progressão do tumor ainda é uma questão controversa devido a multiplicidade de trabalhos presentes na literatura com desfechos diferentes (Bu et al., 2015; Clayton et al., 2011). Nesse contexto, o presente estudo propõem um protocolo para isolar VEs e demonstra que essas VEs derivadas da linhagem celular C6 de GBM são capazes de diminuir o crescimento de GBM tanto *in vitro* quanto *in vivo*, promovendo uma mudança no microambiente tumoral.

Até o presente momento, não há um método padrão de isolamento, detecção e caracterização de VEs. Isso é devido, possivelmente, ao fato que as VEs são partículas nanométricas difíceis de serem isoladas e detectadas. Já existem várias técnicas para o isolamento de VEs de diferentes fontes biológicas, como plasma, urina e sobrenadante de cultura celular com protocolos baseados em centrifugações diferenciais (com ou sem gradientes de densidade), cromatografia por exclusão de tamanho, ultrafiltração, kits (Exo-quick), entre

outros (Théry et al., 2006). Em geral, são utilizadas mais de uma dessas técnicas no mesmo protocolo, na busca de um isolado mais puro. Como grande parte das pesquisas nessa área, utilizamos um protocolo baseado em ultracentrifugação para o isolamento das vesículas obtidas a partir do sobrenadante de cultura celular. Nesse contexto, um protocolo baseado unicamente em centrifugações diferenciais já é considerado um método efetivo na obtenção de VEs homogêneas, principalmente quando originado de amostras menos complexas, como sobrenadantes de cultura, além de ser um protocolo de baixo custo. Por outro lado, esse é um protocolo extenso que não permite o processamento de diversas amostras concomitantemente devido a limitação dos rotores utilizados nas ultracentrífugas, além de necessitar grande volume inicial das amostras.

Como comentado previamente, ainda não há uma padronização para o isolamento de VEs. Do ponto de vista prático, isolar e classificar apropriadamente essas vesículas é primordial para a obtenção de isolados homogêneos e com alta pureza. A falta de padronização pode acarretar interpretações de resultados equivocados, principalmente em VEs isoladas de fluídos corpóreos, como sangue e urina, uma vez que essas fontes apresentam propriedades químicas e físicas muito distintas umas das outras. Em busca de um protocolo apropriado, a Sociedade Internacional de Vesículas Extracelulares (ISEV), desde 2014, fornece os requisitos experimentais mínimos para o isolamento e caracterização das VEs, como formas de isolamento e caracterização. Em nosso estudo, dentre todas as variações testadas, o tempo de descongelamento se demonstrou um passo decisivo na técnica final, em que um descongelamento mais rápido, seguido por um ciclo de centrifugação, resultou em partículas menores e com uma boa concentração de proteína (tabelas I e II). O mesmo foi observado por Trummer e colaboradores (2009), em que as amostras descongeladas em gelo apresentaram significante perda de VEs e mudança na expressão de antígenos quando comparadas a amostras

não congeladas e descongeladas a 37ºC e 25ºC. Essa diferença relativa ao descongelamento no gelo pode estar relacionada com a instabilidade e tendência a agregação das VEs.

Relativo às principais propriedades analisadas na caracterização das VEs, estão tamanho, número e conteúdo. Em relação ao tamanho, a Microscopia Eletrônica de Transmissão (MET) é considerado o método padrão para a visualização de partículas nanométricas, como as VEs, provendo uma indicação da morfologia vesicular e revelando a presença de partículas não-vesiculares; contudo, é um método incapaz de mensurar a contaminação da amostra com fatores solúveis, como proteínas (Chuo, Chien, & Lai, 2018). Para estabelecermos o melhor método de isolamento das VEs, também foram avaliados fatores como tamanho e polidispersão das amostras por meio dos equipamentos ZetaSizer e Nanosight. Na figura 1, podermos observar que os resultados obtidos pelo ZetaSizer são uma combinação de todas as partículas presentes no isolado em uma distribuição monomodal. Considerando que o isolado é uma suspensão de diferentes partículas com distintos tamanhos, o perfil das VEs é amplamente influenciado por partículas maiores, uma vez elas dispersam e espalham mais luz. Dessa forma, por meio do ZetaSizer nós podemos observar uma variabilidade maior entre diferentes análises, já que as subpopulações presentes na amostra podem variar entre os isolamentos (Chu, 2007). O equipamento Nanosight, por outro lado, é capaz de separar diferentes subpopulações, resultando em uma distribuição bimodal, sendo um método mais sensível e o mais utilizado entre pesquisadores para análise de VEs (Vestad et al., 2017). A tabela III apresenta a reprodutibilidade e polidispersão pelos índices D10, D50 e D90, os quais indicam a porcentagem das partículas abaixo desse índice em termos de tamanho. Dessa forma, como mostrado nas figuras 1A e 1B, as VEs apresentam tamanhos menores que 200 nm. EXOs, um dos subtipos de VEs existentes, apresentam tamanhos de 30 – 150 nm, portanto nossa amostra não é composta apenas por EXOs, apresentando partículas maiores. Entretanto, segundo a tabela III, cerca de 50% das VEs são menores que 150 nm, caracterizando nossa

amostra como enriquecida por EXOs. Por fim, as imagens obtidas por MET, demonstram que as VEs foram isoladas com sucesso, apresentam a morfologia clássica e de forma homogênea. Em relação às imagens obtidas pela MET, a morfologia clássica ("*cup-shaped*") das vesículas observadas por meio dessa técnica é devido ao processo convencional de preparação da amostra para a MET, na qual as VEs são desidratadas, colapsando. O uso de outras técnicas para observação dessas VEs, como a microscopia crioeletrônica, permite a observação de vesículas arredondadas.

A estabilidade das VEs também foi avaliada, observou-se que elas se mantêm estáveis em termos de tamanho por pelo menos 18 dias quando armazenadas a 4ºC. Já há na literatura dados relativos a como o método de armazenamento pode afetar o rendimento, composição e função das VEs (Ge et al., 2014; Welch et al., 2017; Zhou et al., 2006). Kalra et al. (2013) analisou por MET VEs derivadas de plasma humano armazenadas a 37ºC, 4ºC, -20ºC e -80ºC por 30 e 90 dias e os melhores resultados obtidos foram a baixas temperaturas (Kalra et al., 2013). Em contrapartida, não há tanta informação relacionada ao armazenamento e estabilidade de VEs derivadas sobrenadante de cultura celular. A ISEV recomenda que as VEs sejam armazenadas a -80ºC em PBS (Witwer et al., 2017). Capricor Therapeutics, em um processo de patente, demonstrou que o tamanho e concentração das VEs se mantém estáveis após uma semana de armazenamento a 4ºC, -20ºC e -80ºC; contudo, os níveis de miRNA diminuem durante esse período quando armazenadas a 4ºC e - 20ºC (Kreke et al., 2015). Em geral, esses dados concordam que a melhor forma de armazenar as VEs é a -80ºC; entretanto, considerando a perda de vesículas durante o processo de congelamento e descongelamento, para experimentos rápidos, o armazenamento a 4ºC pode ser útil e efetivo, pelo menos para o sobrenadante derivado de cultura celular.

Como resultado da sua biogênese, VEs derivados de diferentes tipos celulares são enriquecidas de proteínas de membrana semelhantes à sua célula de origem. Alguns marcadores estão presentes na literatura para caracterizar EXOs em diferentes populações de VEs. Como alguns exemplos de marcadores, temos as tetraspaninas (CD9, CD63, CD81), proteínas envolvidas na apresentação de antígenos (MHC I e II), além de outra variedade de proteínas (TSG101, Alix, anexina, HSP70) (Brinton, Sloane, Kester, & Kelly, 2015; Johnstone, 2006; Clotilde Théry, Zitvogel, & Amigorena, 2002; Trams et al., 1981). A composição proteica das VEs pode ser analisada por diversas técnicas, como proteômica, citometria de fluxo e Western Blot. Essa composição reflete o tipo de célula de origem e a origem endossomal, bem como sua função fisiológica (Niel et al., 2006). Após determinar o melhor método de isolamento das vesículas, nós demonstramos que nosso isolado é rico em VEs com a presença de um marcador clássico de EXOs, como a CD9. De acordo com a ExoCarta, esse marcador é o mais frequente em EXOs (Keerthikumar et al., 2016).

Ainda referente à caracterização, observamos que essas vesículas são capazes de converter o ATP em adenosina (ADO) (Figura 2B). A presença das enzimas CD39 e CD73, essenciais na conversão ATP-ADO, também foi confirmada por citometria de fluxo (Figura 2A), levando-nos acreditar que as VETs são, pelo menos em parte, responsáveis pela produção de ADO extracelular, como mostrado por outros pesquisadores (A. Clayton et al., 2011; Schuler et al., 2014). A principal fonte de ADO no microambiente é resultante do fato das células tumorais superexpressarem a enzima CD73, enquanto células T regulatórias (Treg) superexpressam a enzima CD39. ADO acumulada no microambiente promove crescimento tumoral, angiogênese e inibe respostas antitumorais por meio da supressão de células T efetoras (Teff) e ativação de funções supressoras de células Treg (Deaglio et al., 2007; Magis Mandapathil et al., 2010; Ohta et al., 2006). Dessa forma, o acúmulo de ADO pode induzir um papel pro-tumoral aos VETs, contribuindo para a imunossupressão do microambiente tumoral,

promovendo crescimento tumoral e evasão do sistema imune. Em contrapartida, nossos resultados *in vitro* e *in vivo* demonstraram o oposto. A ADO acumulada no microambiente tumoral pode ser convertida a inosina pela ação da adenosina deaminase (ADA) ou entrar nas células por meio de transportadores. Dessa forma, ADA também apresenta um importante papel na modulação imune, sendo de vital importância para a manutenção da proliferação de Teff (Climent et al., 2009), preservando atividades antitumorais. Ademais, Tregs não expressam CD26 (presente em linfócitos a qual ancora ADA) e, em pacientes com câncer, a expressão do complexo ADA/CD26 está diminuída, o que facilita o tumor a evasão do sistema imune (Magis Mandapathil et al., 2012). Já foi observado que ADA, bem como transportadores de nucleosídeos (em especial o ENT2), estão expressos em células C6 (Ohkubo, Nagata, & Nakahata, 2007; Sinclair et al., 2002), dessa forma, podemos acreditar que eles também estejam expressos nas vesículas derivadas dessas células, o que pode justificar, pelo menos em parte, o papel antitumoral das VEs observado em nosso estudo.

Devido ao fato das VEs interagirem e modularem os linfócitos, além de conter moléculas supressivas e estimulantes semelhantes a sua célula de origem, como antígenos associados ao tumor, características genômicas tumor-específicas, leva-nos a pensar que o fato imunogênico está muito mais associado com a atividade antitumoral observada em nossos resultados que a imunossupressão promovida pela ADO. Nossos resultados *in vitro* de viabilidade e *in vivo* mostram um grande potencial antitumoral relacionado às VEs, isso sugere que deve haver outros fatores vesiculares que contribuem na ativação da resposta imune anti-tumoral. Além disso, estudos já demonstraram que a função biológica, bem como o conteúdo das VEs são dependentes da origem da célula (W. Chen et al., 2014; Wieckowski & Whiteside, 2006). No campo da imunoterapia, a aplicação de VEs originalmente estava baseada em VEs derivadas de células dendríticas (DC), ricas em CD9, CD81 e MHC I e II, capazes de induzir resposta imune antitumoral (Delcayre, Shu, & Pecq, 2005). Já existem, entretanto, estudos

envolvendo VETs que, apesar da já conhecida atividade imunossupressora por meio da inibição da atividade de Teff,, ativação da atividade supressiva de Treg e comprometida atividade das células NK (Clayton et al., 2007; Zhang et al., 2007), suportam que a resposta imune induzida pelas proteínas de choque térmico presentes nas VEs são capazes de promover ativação de células NK, lise do tumor pela liberação de granzima B e indução de resposta T citotóxica antígeno-específica (Dai et al., 2005; Khalil, Kabapy, Deraz, & Smith, 2011; Yufeng Xie et al., 2010).

Além disso, VETs são fontes de antígenos tumorais. Nosso estudo mostrou uma significante redução do tumor após o tratamento com VETs e, adicionalmente, uma mudança do perfil do microambiente tumoral de pro-tumoral para anti-tumoral, com uma importante redução de células Treg imunossupressoras. Além do fato da redução de células Treg estar relacionado com a redução tumoral, o exato mecanismo por trás desse resultado ainda não é bem compreendido, entretanto, nós acreditamos que os VETs carregam materiais antigênicos e MHC, que são fundamentais no processo de apresentação de antígeno, promovendo uma forte atividade de células Teff e suprimindo Treg. Wolfers et al. (2001) acredita que VETs são uma via importante para a apresentação cruzada de antígenos devido a forma como ocorre a biogênese dessas vesículas, permitindo que elas sejam enriquecidas de moléculas (MHC, HSP70) e antígenos tumorais nativos capazes de desencadear uma resposta antitumoral (Wolfers et al., 2001). Já foi observado que VEs derivadas tanto de tumores quanto de CD são capazes de transferir antígenos para CD e desencadear uma efetiva ativação de células T *in vitro* e *in vivo*, levando a rejeição de tumores autólogos (Andre et al., 2002; Wolfers et al., 2001). Alguns estudos também relacionam o papel imunogênico de VETs com a liberação de citocinas pró-inflamatórias, como IL2 e IL18 (Dai et al., 2006; Yang et al., 2007), ativação de CD (Chen et al., 2011) e geração de linfócitos T citotóxicos específicos (Altieri et al., 2004) . Tais estudos ajudam a suportar nossos achados, pelo menos os relativos ao modelo *in vivo*.

Nosso modelo de coinjeção de TEVs se mostrou mais efetivo no desencadeamento da resposta imunológica no microambiente tumoral em relação as células Treg, havendo uma significante redução dessas células. Acreditamos que esse fato se deve, pelo menos em parte, ao fato das vesículas estarem sendo injetadas no momento do implante tumoral, o que pode influenciar desde o princípio toda dinâmica tumoral. Marabelle et al. (2013) demonstrou, ao depletar Tregs por meio de injeções intratumorais com imunomoduladores, uma diminuição do crescimento tumoral, bem como ausência de metástases (Marabelle et al., 2013). O pré-tratamento dos ratos com VETs também promoveu uma diminuição do tumor; contudo, não houve diferença em relação a células regulatórias. Como o pré-tratamento busca estimular o sistema imune previamente ao implante do tumor, acreditamos que o mecanismo por trás da redução do tumor no grupo dos animais pré-tratados esteja relacionado com células $CD8^+$ de memória. Em relação a imunoterapia, a geração de células T de memória contra antígenos tumorais é fundamental e ocorre com auxílio de citocinas (IFN-α e IL12, por exemplo), as quais fornecem sinais que ativam as T CD8+, induzindo a um fenótipo de memória (Mescher et al., 2007).

Apesar de existirem estudos relatando a relação de VEs derivados de glioma (VEGs) com a imunossupressão do microambiente e consequente progressão tumoral, (Domenis et al., 2017; Hellwinkel et al., 2016; Iorgulescu et al., 2016), ainda há poucos estudos mostrando o efeito anti-tumoral de VEGs (Bronisz et al., 2014; Katakowski et al., 2013; Muller et al., 2015). Até presente momento, acreditamos que esse seja o primeiro estudo demonstrando que as VEs derivadas da linhagem C6 de rato seja capaz de suprimir o crescimento de GBM *in vivo* e modular o microambiente tumoral. Mostramos também que a forma de tratamento também influencia a resposta imunológica desencadeada, uma vez que o modelo de coinjeção, ao atuar mais localmente, reduziu células regulatórias, ao passo que o modelo de imunização não afetou essas células. Presumimos que essa resposta possa relacionada a formação de células $CD8^+$ de

memória, induzindo a uma resposta imunológica tumor-específica. Além disso, dados preliminares do grupo, não mostrados no presente trabalho, mostraram que o tratamento após o implante tumoral não promoveu redução do tumor, tornando claro o papel das VEs em ativar uma resposta imunológica contra o tumor.

5 CONCLUSÃO

Neste trabalho, propomos um protocolo para isolamento de vesículas extracelulares derivadas da linhagem C6 de GBM, além de caracterizá-las de acordo com a presença de marcadores como CD9, CD39 e CD73. Além disso, demonstramos que essas vesículas desempenham um papel antitumoral na progressão do GBM *in vitro* e *in vivo*, reduzindo a proliferação tumoral e a presença de linfócitos T regulatórios no microambiente tumoral.

6 PERSPECTIVAS

- Realização da técnica de Western Blot para CD9, HSP70, CD39 e CD73;

- Realização de tratamento após o implante do tumor e posterior avaliação do tamanho e microambiente tumoral;

- Avaliação do microambiente tumoral em relação a células $CD8^{+}$, NK, bem como a produção de citocinas;

- Isolar VEs provenientes do sangue periférico dos ratos submetidos às diferentes modalidades de tratamentos, buscando possíveis biomarcadores.

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ANEXO A: Carta de aprovação do CEUA

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA
Comissão De Ética No Uso De Animais

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 33505
Título: AVALIACAO DO PAPEL DESEMPENHADO POR EXOSSOMOS DERIVADOS DE CELULAS DE GLIOMA NA MODULACAO DE LINFOCITOS PERIFERICOS E NA PROGRESSAO TUMORAL

Vigência: 01/07/2017 à 31/07/2019

Pesquisadores:

Equipe UFRGS:

ANA MARIA OLIVEIRA BATTASTINI - coordenador desde 01/07/2017
Fabrício Figueiró - pesquisador desde 01/07/2017
Juliete Nathali Scholl - Aluno de Mestrado desde 01/07/2017

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 25/09/2017 - SALA 330 DO ANEXO - PRÉDIO DA REITORIA DA UFRGS/CAMPUS CENTRO/UFRGS, em seus aspectos éticos e metodológicos, para a utilização de 70 ratos machos Wistar de 8-9 semanas (220-300g) provenientes do Biotério do Departamento de Bioquímica da UFRGS, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Quinta-Feira, 5 de Outubro de 2017

MARCELO MELLER ALIEVI
Coordenador da comissão de ética

ANEXO B: Colaborações (Artigos publicados no período do mestrado)

1) Azambuja, J. H., Gelsleichter, N. E., Beckenkamp, L. R., Iser, I. C., Fernandes, M. C., Figueiró, F., Battastini, A.M.O; **Scholl, J.N**; de Oliveira, F.H; Spanevello, R.M; Sévigny, J; Wink, M.R; Stefani, M. A; Teixeira, H.F; Braganhol, E. (2018). *CD73 Downregulation Decreases In Vitro and In Vivo Glioblastoma Growth*. Molecular Neurobiology. doi:10.1007/s12035-018-1240-4

2) Gardani, C. F. F., Cappellari, A. R., de Souza, J. B., da Silva, B. T., Engroff, P., Moritz, C. E. J., **Scholl, J.N**; Battastini, A.M.O; Figueiró, F.; Morrone, F. B. (2019). *Hydrolysis of ATP, ADP, and AMP is increased in blood plasma of prostate cancer patients. Purinergic Signalling*.doi:10.1007/s11302-018-9642-3